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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE TRADEMARK TRIAL AND APPEAL BOARD

Proceeding	91194218
Party	Plaintiff Illumina, Inc.
Correspondence Address	SUSAN M NATLAND KNOBBE MARTENS OLSON & BEAR LLP 2040 MAIN ST, 14TH FL IRVINE, CA 92614 UNITED STATES efiling@knobbe.com
Submission	Plaintiff's Notice of Reliance
Filer's Name	Susan M. Natland
Filer's e-mail	efiling@knobbe.com
Signature	/susan m. natland/
Date	04/08/2015
Attachments	Opposer's Rebuttal Notice of Reliance ILLINC.266M.pdf(157046 bytes) Exhibit 403 Next Generation Diagnostics.PDF(352323 bytes) Exhibit 404 CDC amd4-2-13 A New Landscape for.PDF(799942 bytes) Exhibit 405 HHRG-113-IF14-Wstate-MertzA-20140909 ACLA Statement of.PDF(811131 bytes) Exhibit 406 UVA Health System.PDF(291856 bytes) Exhibit 407 Next Generation Sequencing _ University of Washington Laboratory Medicine.PDF(596435 bytes) Exhibit 408 Stanford Hospital_ Clinical Laboratory Departments.PDF(102083 bytes) Exhibit 409 Available Tests _ University of Washington Laboratory Medicine.PDF(118130 bytes) Exhibit 410 UCSF Developing NGS based Infectious Disease Dx Tests Metagenomic.PDF(1293373 bytes) Exhibit 411 BTN_A_000112628_O_1545a An oligonucleotide microarray for.PDF(849945 bytes) Exhibit 412 Microarray multiplex assay for.PDF(303127 bytes) Exhibit 413 Basic Concepts of Microarrays and.PDF(490972 bytes) Exhibit 414 DNA microarray analysis of predominant.PDF(739956 bytes) Exhibit 415 DNA Sequencing Says New Hybrid E. Coli Strain Is Cause Of German Outbreak.PDF(1246983 bytes) Exhibit 416 ARUP Exhibit.PDF(404760 bytes) Exhibit 417 Quest Exhibit.PDF(543425 bytes) Exhibit 418 CFR - Code of Federal Regulations Title 21, Volume 8, Revised as of April 1, 2014, Chapter 1 Food and Drug Administration Department of Health and Human Services Subchapter H.PDF(621587 bytes) Exhibit 419 FDA In Vitro Diagnostics _ Laboratory Developed Tests.PDF(562174 bytes) Exhibit 420 FDA Overview of IVD Regulation ucm123682.PDF(1082432 bytes) Exhibit 421 Oxford dictionary definitions Exhibit.PDF(742985 bytes)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE TRADEMARK TRIAL AND APPEAL BOARD

Illumina, Inc.,	)	Opposition No.: 91194218
	)	
Opposer,	)	
	)	
v.	)	
	)	
Meridian Bioscience, Inc.,	)	
	)	
Applicant.	)	
	)	

OPPOSER'S REBUTTAL NOTICE OF RELIANCE

Commissioner for Trademarks
P.O. Box 1451
Alexandria, VA 22313-1451

Dear Sir or Madam:

Pursuant to pursuant to 37 C.F.R. §§ 2.120 and 2.122, Opposer, Illumina, Inc., hereby makes the following of record and notifies Applicant of its reliance on the following rebuttal documents and/or testimony:

Exhibit 403

Website printout from the Seventh Annual Next Generation Diagnostics Summit in Washington, D.C. Exhibit 403 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 404

Brochure from the Centers for Disease Control and Prevention (CDC) titled "A New Landscape for Combatting Infectious Diseases." Exhibit 404 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 405

Statement of Alan Mertz, President, The American Clinical Laboratory Association for U.S. House of Representatives Energy and Commerce Committee Subcommittee on Health

Hearing on 21st Century Cures: Examining the Regulation of Laboratory-Developed Tests. Exhibit 405 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 406

Website printout from University of Virginia Health System's Microbiology and Molecular Diagnostics – Infectious Diseases department. Exhibit 406 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 407

Website printout from the University of Washington Department of Laboratory Medicine's Molecular Diagnosis Microbiology Section titled "About Next Generation 16S Sequencing." Exhibit 407 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 408

Website printout from Stanford Health Care's Pathology & Laboratory Medicine "Anatomic Pathology & Clinical Laboratory Departments." Exhibit 408 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 409

Website printout from the University of Washington Department of Laboratory Medicine's Molecular Diagnosis Microbiology Section. Exhibit 409 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 410

Genoneweb.com article published online on February 20, 2015, titled "UCSF Developing NGS-based Infectious Disease Dx; Tests Metagenomic Sequencing on Minlon." Exhibit 410 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 411

BioTechniques Vol. 44, No. 2, 2008 Research Report titled "An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1,HBV, and HCV. Exhibit 411 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 412

ScienceDirect article published online on March 26, 2007, titled "Microarray multiplex assay for the simultaneous detection and discrimination of hepatitis B, hepatitis C, and human immunodeficiency type-1 viruses in human blood samples." Exhibit 412 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 413

Clinical Microbiology Reviews, Oct. 2009, p. 611-633, Vol. 22, No. 4 article by Melissa B. Miller and Yi-Wei Tang titled "Basic Concepts of Microarrays and Potential Applications in Clinical Microbiology." Exhibit 413 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 414

Elsevier's Molecular and Cellular Probes 18 (2004) 223-224 article by Rong-Fu Wang, Marjorie L. Beggs, Bruce D. Erickson, Carl E. Cerniglia titled "DNA microarray analysis of predominant human intestinal bacteria in fecal samples." Exhibit 414 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 415

Science20.com article published online on June 2, 2011, titled "DNA Sequencing Says New Hybrid E. Coli Strain is Cause of German Outbreak." Exhibit 415 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 416

Website printouts from ARUPlab.com titled "About ARUP," "Myeloid Malignancies Mutation Panel by Next Generation Sequencing," "Organism Identification by 16S rDNA Sequencing," ""*Clostridium difficile* toxin B gene (tcdB) by PCR" and "*Streptococcus* Group B by PCR." Exhibit 416 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 417

Website printouts from SpecialtyLabs.com (Quest Diagnostics Nichols Institute of Valencia) titled "About Us," "Contact Us," "Test Menu," "17221: Bacterial 16s rDNA Sequencing," "16377: Clostridium Difficile Toxin B, Qualitative Real-Time PCR" and "91427: Melanoma, Chromosomal Microarray, Clarisure® Oligo-SNP." Exhibit 417 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 418

Code of Federal Regulations (CFR) Title 21, Volume 8, Revised as of April 1, 2014, Chapter 1 Food and Drug Administration Department of Health and Human Services Subchapter H – Medical Devices –Part 864 Hemotology and Pathology Devices – Subpart E – Specimen Preparation Reagents, Sec. 864.4010 General purpose reagent. Exhibit 418 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 419

Website printout from the U.S. Food and Drug Administration (FDA) titled "Laboratory Developed Tests." Exhibit 419 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 420

Website printout from the U.S. Food and Drug Administration (FDA) titled "Overview of IVD Regulation" which provides an overview of how FDA regulates IVDs. Exhibit 420 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 421

Website printout from OxfordDictionaries.com defining IMMUNO-., TRUE, PROFESSIONAL, PRO and GENE. Exhibit 421 is relevant generally to show the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

The above described documents are filed concurrently herewith.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: April 8, 2015

By: _____

Susan M. Natland
Brian C. Horne
Marissa Calcagno
Hans L. Mayer
Knobbe Martens Olson & Bear, LLP
2040 Main Street, 14th Floor
Irvine, California 92614
efiling@knobbe.com
Tel: (949) 760-0404
Fax: (949) 760-9502
Attorneys for Opposer, Illumina, Inc.

CERTIFICATE OF SERVICE

I hereby certify that I served a copy of the foregoing Opposer's Rebuttal Notice of Reliance upon Applicant's counsel by depositing one copy thereof in the United States Mail, first-class postage prepaid, on April 8, 2015, addressed as follows:

J. Michael Hurst
Keating Muething & Klekamp PLL
One East 4th Street
Suite 1400
Cincinnati, OH 45202

Sarah Beno Couvillion

20352314

Exhibit 403

Seventh Annual
Next Generation Dx SUMMIT
August 18 - 20, 2015 | Capital Hilton Hotel | Washington, DC
Moving Assays to the Clinic

Archived Content

As the need for faster, more accurate, and effective molecular-based tests grows, Cambridge Healthtech Institute's 6th Annual **Molecular Diagnostics for Infectious Disease** conference showcases cutting-edge technologies and tests being brought into routine clinical practice for rapid pathogen detection and identification. Several technologies and tests featured include next-gen sequencing, GI panels, and molecular-based tests for sepsis and blood borne infections. Outcome studies measuring the impact of the diagnostic on patient health and outcome, critical to the successful adoption of these molecular diagnostics in the clinic, will be discussed.

Day 1 | **Day 2** | **Short Courses** | **Download Brochure**

WEDNESDAY, AUGUST 20

10:30 am Registration

PLENARY SESSION: THINK TANK ON NEXT-GENERATION SEQUENCING DIAGNOSTICS

11:00 Chairperson's Opening Remarks
Harry Glorikian, Healthcare Consultant

11:10 Discussion: Regulatory Review of Clinical Sequencing Assays

Moderator: Harry Glorikian, Healthcare Consultant

Guest Speaker: Jennifer Dickey, RAC, Ph.D., Office of In Vitro Diagnostics, DIHD, US Food and Drug Administration

In November of 2013, the FDA issued the first clearances of Next Gen Sequencing- (NGS) based assays. There have additionally been a number of clinical trials approved recently that utilize NGS-based assays for patient enrollment or stratification. In light of the expanding roles that new sequencing technologies are playing in clinical decision making, this talk will focus on critical elements that FDA considers when evaluating NGS validation using the recent clearances/approvals as examples. There will also be a discussion of any new communications that FDA has issued in regard to the regulatory review of NGS- based assays. Following the discussion there will be a Q&A with the audience.

11:55 Next-Generation Sequencing in Clinical Practice: Case Reports of

Co-Organized with

2014 Brochure

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Clinical Utility and Reimbursement

Moderator: Elaine Lyon, Ph.D., Medical Director, Molecular Genetics, ARUP

Case Presenters:

Andrea Ferreira-Gonzalez, Ph.D., Professor, Pathology; Director, Molecular Diagnostics Lab, Virginia Commonwealth University

Madhuri Hegde, Ph.D., FACMG, Professor, Human Genetics; Executive Director, Emory Genetics Laboratory, Emory University School of Medicine

The landscape of next-generation sequencing diagnostics is changing rapidly. Clinical laboratories are offering highly complex tests using new technologies, but face challenges in reimbursement. To be reimbursed for these tests, laboratories will need to address clinical utility as well as clinical validity. Clinical cases that demonstrate the utility of genomic oncological and inherited disease testing will be presented. Experiences with reimbursement of these tests will be discussed.

12:40 pm Enjoy Lunch on Your Own

OUTCOME STUDIES & CLINICALLY ACTIONABLE DIAGNOSTIC ASSAYS FOR PATHOGENS PART I

1:50 Chairperson's Opening Remarks
Nathan A. Ledeboer, Ph.D., D(ABMM), Assistant Professor & Medical Director, Clinical Microbiology, Medical College of Wisconsin

2:00 Impact of Molecular Diagnostics on Infection-Related Patient Outcomes

Jerod Nagel, Pharm.D., BCPS (AQID), Clinical Specialist, Infectious Diseases, Clinical Assistant Instructor, Director Infectious Diseases Residency, University of Michigan Hospital and Health Systems, University of Michigan, College of Pharmacy
The advancement of molecular diagnostics has improved the ability to identify pathogens and resistance mechanisms in a timely manner. Demonstrating the impact on patient outcomes and overall hospital expenditure should be an important component in deciding the role of molecular diagnostics in clinical laboratories. This presentation aims to review the outcomes associated with new diagnostics and discuss strategies to optimize patient outcomes.

2:30 Unbiased Next-Generation Sequencing — Moving Towards Clinically Actionable Diagnostic Assays for Pathogens

Charles Chiu, M.D., Ph.D., Assistant Professor, Lab Medicine and Medicine, Infectious Diseases, University of California San Francisco
Unbiased next-generation sequencing technology enables the detection of novel or uncommon pathogens directly from clinical samples, but its routine implementation in clinical and public health settings has been hindered by issues of cost, turnaround time, and bioinformatics analysis of complex datasets. Here we will describe the use of the technology in the clinical laboratory to validate novel, ultra-sensitive assays that have the potential to transform infectious diseases diagnosis, including a case where NGS rapidly identified a rare pathogen leading to a dramatic, real-time impact on the care and treatment of an immunocompromised child with a fulminant, unknown encephalitis.

GI PANELS: EMERGING TECHNOLOGIES AND EFFECTIVENESS IN PATIENT CARE AND OUTCOME

2:50 From Culture to the Future: Molecular Detection of Enteric Pathogens

Nathan A. Ledeboer, Ph.D., D(ABMM), Assistant Professor & Medical Director, Clinical Microbiology, Medical College of Wisconsin
The diagnosis of enteric pathogens is particularly challenging given the large amount of vastly diverse indigenous gastrointestinal flora present in stool. Stool cultures have low sensitivity due to variable amounts of pathogen shedding and obscuring normal flora.

Studies have shown a diagnostic yield of stool culture as low as 1.5%, with a cost per positive culture as high as $1,200. Molecular methods boast increased sensitivity and specificity when compared to stool culture. Several molecular methods including real-time PCR, microarray, and liquid array assays have been described. This presentation will evaluate the performance of molecular gastroenteritis assays and begin to evaluate the clinical and cost saving benefits.

3:10 The Use of Molecular Stool Panels in the Diagnosis of Infectious Gastroenteritis

Susan M. Novak-Weekley, Ph.D., D(ABMM), Director, Microbiology, Kaiser Permanente, SCPMG Regional Reference Laboratories

Infectious gastroenteritis is a major public health concern. The landscape is changing within the clinical laboratory setting in regards to diagnosis of bacterial, viral and parasitic stool pathogens. Several manufacturers are developing multiplex molecular panels for the diagnosis of these pathogens. This session will discuss the newer panels coming out on the market and performance data related to those assays. How these panels will fit into the laboratory and clinical setting will also be discussed.

3:30 Dual Site Clinical Evaluation of the xTAG Gastrointestinal Pathogen Panel for Detection of Infectious Gastroenteritis

Anami Patel, Ph.D, MB (ASCP) DLM, Technical Director, Molecular Diagnostics Laboratory, Le Bonheur Children's Hospital

We evaluated the clinical performance and laboratory cost and time efficiencies gained through use of the xTAG gastrointestinal pathogen in vitro diagnostic (IVD) assay in a comparison between clinical and public health laboratories. The site reproducibility study showed 98.7% agreement with high positive and negative agreement values (96.2% and 99.8%, respectively). High throughput detection of multiple GI pathogens improved turnaround time, consolidated laboratory workflow, and simplified stool culture practices, thus reducing the overall cost and number of specimens processed.

4:00 Refreshment Break in the Exhibit Hall with Poster Viewing

USE OF NEXT-GEN SEQUENCING TECHNOLOGIES IN PATIENT CARE & OUTCOME WITH ID

4:45 From Genome to Biomarker: The Path Forward

David A. Rasko, Ph.D., Associate Professor, Microbiology & Immunology, University of Maryland School of Medicine Institute for Genome Sciences

The advent of more decreased sequencing costs and increased throughput have allowed movement beyond the examination of prototype isolates and into the use of microbial genomic data for other purposes. Microbial pathogens are identified based on virulence factors that are often encoded on mobile elements, and thus have variable presence or absence. Identification of stable genomically derived biomarkers will allow more robust identification of bacterial pathogens. Genomic sequencing is opening this avenue of biomarker development.

5:15 Next-Generation Sequencing for Pathogen Detection - HIVE Platform Solutions and Applications to Viral Detection and Other Bioinformatics Pipelines

Vahan Simonyan, Ph.D., HIVE Project Lead, CBER, FDA

Next-generation sequencing production has exponentially increased in recent years due to the reduction in cost and improvements in performance of the technology. A single sample generally provides ample coverage but, due to the highly complex genomes of many pathogenic organisms, development of improved computational methods and platforms is now necessary to meet the increasing demands of related studies. HIVE, the High-performance Integrated Virtual Environment, is a cloud-based environment specifically developed to meet the growing storage and analysis challenges associated with NGS data and other big data types. The HIVE platform provides secure web access for registered users to store, annotate, analyze and retrieve big data, as well as a visually dynamic interface for viewing computational results. HIVE can be used for pathogen detection through the following applied pipelines: big data management (NGS, mass spec, etc.); alignment of unknown or mixed samples to reference viral genome sets; computation and comparison of SNP profiles of patients or pathogen samples; discovery of recombination events implicated in virulence recovery and pathogenicity; facilitation of collaborative discovery and annotations.

5:45 Novel Methods for Sample Preparation & Target Enrichment in Molecular and Protein Diagnostics for Infectious Disease and Beyond - Lessons from Mass Spec and NGS

Michael Super, Ph.D., Senior Staff Scientist, Advanced Technology Team, Wyss Institute at Harvard

We have developed methods for sample extraction from complex biological environments using broad-spectrum pathogen binding proteins attached to nanomagnetic particles. These have been used to enrich samples for molecular and protein analysis without the need for culturing, thereby saving significant time and providing same-day pathogen identification. An overview of the challenges of sample prep in the current environment of NGS and genomic testing will also be discussed.

6:15 Close of Day

6:00 Dinner Short Course Registration

RECOMMENDED DINNER SHORT COURSE*

6:30-8:30 pm NGS for Infectious Disease Diagnostics - View Detailed Agenda
** Separate registration required*

Day 1 | **Day 2** | **Short Courses** | **Download Brochure**

Cambridge Healthtech Institute
Your Life Science Network
250 First Avenue Suite 300
Needham, MA 02494
P: 781.972.5400
F: 781.972.5425
E: chi@healthtech.com

Exhibit 404

A New Landscape for Combatting Infectious Diseases

Advances in science and technology aimed at identifying the complete genetic makeup of microorganisms are ushering in a new era for controlling infectious threats. By using genetic sequencing to examine infectious pathogens, these technologies are on the verge of revolutionizing our ability to diagnose infectious diseases, investigate and control outbreaks, understand transmission patterns, develop and target vaccines, and determine antimicrobial resistance—all with increased timeliness and accuracy and decreased costs. Recent reports have highlighted early efforts on the use of whole-genome sequencing (WGS) technologies in investigating outbreaks of drug-resistant bacteria in hospitalized patients at the National Institutes of Health Clinical Center and in the United Kingdom (**Box 1**), an outbreak of foodborne disease in Europe, and an outbreak of tuberculosis in Canada. Termed "genomic epidemiology," this approach to infectious disease control was named one of the six **"Areas to Watch in 2012"** by the journal *Science* on the basis of its transformative potential in **"determining quickly where newly emerging diseases come from, whether microbes are resistant to antibiotics, and how they are moving through a population."**

New opportunities bring new challenges

Along with the many opportunities afforded by these "next-generation" sequencing technologies come new responsibilities and challenges for clinical and public health. In particular, the availability and ease-of-use of WGS technology is rapidly changing the way that diseases are studied—providing a new level of detailed information to better understand how infections occur and are transmitted.

The major benefit of genomic sequencing is its ability to rapidly generate massive amounts of data on a pathogen. **A key component in the usefulness of these technologies, however, is ensuring the availability of sufficient laboratory and computing infrastructure (i.e., "bioinformatics") and highly skilled experts to manage, analyze, evaluate, and gain new information from (i.e., make sense of) these large amounts of biological data**.

CDC is requesting dedicated FY 2014 funding to develop and incorporate new molecular technologies and bioinformatics capacities into public health—improving infectious disease control efforts by CDC and its public health partners. CDC's current capacities in these areas are not sufficient to meet the challenges of this rapidly evolving field, threatening the Agency's ability to meet its public health mission.

Ensuring continued public health capacity at CDC

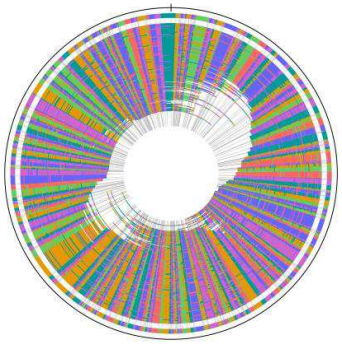
The Centers for Disease Control and Prevention (CDC) is recognized and relied upon nationally and globally for its strong epidemiologic and laboratory expertise and capacity for controlling infectious diseases, with laboratory reference capability for virtually all human infections. Ensuring that CDC can continue to meet its responsibilities is a critical public health priority. Because of resource constraints, CDC has not been able to develop the bioinformatics capacity to keep pace with the rapidly growing field of molecular diagnostics.

To better identify gaps and make recommendations for best use of Agency resources in meeting these challenges, CDC convened a panel of external experts in bioinformatics, informatics, and laboratory information technology in 2011 to review the status of this technology at CDC and its ability to meet programmatic needs. Among their feedback was a clarion call for urgent action:

> *CDC is known for its strong ability to accurately analyze and interpret disease surveillance data. However, the Agency runs the risk of going from* ***outdated to obsolete and then to irrelevant*** *without strong investments in the science of bioinformatics . . .*

Expanding molecular technologies and bioinformatics capacities at CDC

The expert panel recommended both short-term and long-term goals to meet the most urgent needs and opportunities and to ensure that CDC's capacities in these areas become "**sustainable resources for public health**." CDC has worked to address several of the short-term goals, including developing a nascent bioinformatics program to provide initial support for evolving scientific needs. Among its activities, this core group has worked with programs across CDC's infectious disease laboratories to identify priorities and to strengthen collaborations with outside agencies and partners. Examples of priority areas include the following:



Genomic mapping of *Legionella pneumophila* serogroup 8

- **Bioinformatics Program Development**
  - Enhancing CDC's bioinformatics expertise and infrastructures
  - Working with partners to establish bioinformatics training fellowships to meet future workforce needs
- **Next-Generation PulseNet (Box2)**
  - Modernizing PulseNet to encompass whole genome sequencing technologies to improve our ability to detect and respond to foodborne disease outbreaks
  - Expanding the use of these new capacities to other CDC national infectious disease surveillance networks
- **The Research Grade Network**
  - Establishing a separate information technology network for use by CDC laboratories and their public health partners
  - Ensuring broader access to and facilitating rapid exchange of laboratory and bioinformatics information and datasets
- **MicrobeNet**
  - Creating a system of web-accessible, searchable databases containing detailed, reference information to characterize infectious pathogens
  - Ensuring near real-time analysis and feedback of information on submitted pathogens for CDC and its local, state, national, and international laboratory partners

Now and into the future

Molecular-based technologies represent an evolving and rapidly changing field. While the potential for their use across a wide range of specialties continues to evolve, at present they are on a course to totally reshape the practices of microbiology and infectious disease control. As the use of these technologies extends to more and more clinical and laboratory settings, it is critical for CDC to have the molecular tools and bioinformatics capacity to provide expertise and leadership in ensuring that the expanding use of these technologies translates into meaningful information to guide effective public health action.

Advances in molecular-based technologies along with enhanced bioinformatics capacities ***generate a new level of information on infectious pathogens*** *that can be used to more accurately and rapidly*

- *Diagnose infectious diseases*
- *Investigate and control outbreaks*
- *Understand transmission patterns*
- *Determine antimicrobial resistance*
- *Develop and target prevention measures, including vaccines*

It is critical for CDC to have the molecular tools and bioinformatics capacity to provide expertise and leadership in ensuring that the expanding use of these technologies translates into effective information for guiding public health action.

ILLUM-3835

A New Landscape for Combatting Infectious Diseases

Box 1. New Approaches to Reducing Healthcare-Associated Infections and Detecting Antibiotic Resistance

More than 1 million healthcare-associated infections (HAIs) and 100,000 related deaths occur in the United States each year. At any given time, approximately 1 in 20 patients receiving treatment in U.S. hospitals has an HAI. In addition to their tremendous toll on human health, these largely preventable infections add billions of dollars to healthcare costs each year. Although much progress towards reducing these infections has been made over the last several years, HAIs remain all too common. Complicating control efforts is the increasing number of outbreaks that involve drug-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile*, and even more difficult-to-treat, gram-negative bacterial infections such as *Escherichia coli* and *Klebsiella pneumoniae*. These gram-negative infections are increasingly resistant to most available antibiotics and can also pass along genetic materials that enable other bacteria to become drug-resistant. With new resistant strains being identified across a spectrum of healthcare-associated, foodborne, respiratory, STD, and other infections, advances in genomic sequencing can play a critical role in rapidly identifying these infections, tracking their spread, and improving control measures.

Use of WGS in Investigating Outbreaks of HAIs

Whole-genome sequencing (WGS) technology has been used to investigate several outbreaks of drug-resistant HAIs. For one investigation, researchers in the United Kingdom used whole-genome sequencing to re-examine an outbreak of MRSA that had occurred earlier in a neonatal unit. Because traditional methods of subtyping are not able to clearly identify related MRSA infections, the researchers looked to WGS to better define the infections and determine transmission linkages. The data identified a cluster of associated infections as well as separate infections not linked to the outbreak. Although this investigation was performed retrospectively, it highlighted the potential use of WGS in providing timely and highly accurate information to better guide patient care and to improve infection control.

Another recent investigation involved the use of WGS to help investigate the spread of carbapenem-resistant *K. pneumoniae* among patients at the National Institutes of Health Clinical Center, after introduction of the highly resistant infection from a woman transferred to the center to participate in a clinical trial in 2011. Despite intensive infection control efforts, including almost immediate isolation of the index patient, the bacteria began to appear in patients across other areas of the center. Many of these individuals died—some from their underlying illness and others from the infection. Again, current typing methods are not able to distinguish differences in *K. pneumoniae*, so it was unclear whether all the patients were infected with the same strain. Medical staff reached to researchers at the National Human Genome Research Institute, who used genomic sequencing to help determine how the infection spread. The sequencing showed that all of the cases likely originated from the index patient, with the infections transmitted from bacteria on at least two different sites on her body during at least three separate events—providing important information for changing and implementing new infection control measures.

Advances in genomic sequencing can play a critical role in rapidly identifying HAIs and tracking their spread—providing important information for improving control measures. CDC's extensive and highly successful infectious disease surveillance networks, involving clinical, laboratory, and public health partners across the nation and globally, are poised to leverage these technologies in new and exciting ways to improve public health.

Leveraging Molecular Technologies to Advance Public Health

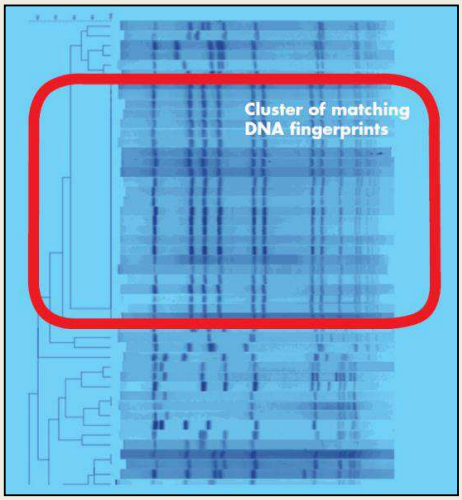
CDC has strong expertise in the areas of HAIs and antimicrobial resistance. In fact, CDC's leadership in reducing HAIs through targeted educational and infection control efforts; improved means of reporting and tracking infections and evaluating control efforts; and increased involvement of states in these prevention efforts has helped the nation achieve dramatic results in this area, with reductions of nearly 60% in the occurrence of certain types of healthcare-associated bloodstream infections. CDC's extensive and highly successful infectious disease surveillance networks have been developed over the years with clinical, laboratory, and public health partners across the nation and globally. These surveillance platforms are poised to leverage WGS and other molecular technology advancements in new and exciting ways to improve public health.

Box 2. Expanding the Use of Molecular Technologies to Improve Food Safety: *Next-Generation PulseNet*

An estimated 48 million foodborne illnesses occur in the United States each year, resulting in 128,000 hospitalizations and 3,000 deaths. CDC tracks and investigates foodborne diseases in collaboration with state and local health departments and other partners, working to rapidly identify their sources and contain their spread. Essential to these efforts is PulseNet, a network of state and local public health and food regulatory agency laboratories across the United States and internationally that work to identify similar cases of foodborne illnesses that might signal an outbreak. Over the past 15 years, PulseNet has revolutionized our ability to detect clusters of foodborne illness, detecting hundreds of outbreaks, enabling faster and better responses, and leading to important improvements in food safety.

Enabling Real-time Communication on Foodborne Illness

PulseNet's national network of more than 80 state and federal laboratories use pulsed-field gel electrophoresis (PFGE) to examine DNA patterns of bacterial infections—a technology that has been used for decades. These laboratories submit PFGE patterns from bacteria of persons with foodborne infections to a CDC database for rapid comparison of strains of organisms such as *Escherichia coli* O157:H7, *Salmonella*, *Shigella*, *Listeria*, and *Campylobacter*. Each year, approximately 50,000 DNA fingerprints are uploaded into the PulseNet database. Today, **this laboratory network tracks a cumulative database that represents nearly half a million bacterial isolates from food, the environment, and persons with foodborne illness**. In addition to allowing for real-time communication among CDC, state and local health departments, and international partners, PulseNet also helps food regulatory agencies identify areas needing additional food safety measures by linking bacteria causing illnesses in people to bacteria detected in food.



Modernizing PulseNet to Meet the Demands of Next-Generation Sequencing Technologies

Like many of our public health disease surveillance networks, PulseNet relies on isolates from culture-based testing methods, the long-held standard in testing of specimens from patients with foodborne and other infections. However, more and more diagnostic tests that do not use culture are being developed and marketed for clinical use, including use by clinical laboratories in diagnosing foodborne infections. Because of their reduced costs, rapid turnaround times, and less labor-intensive methodology, these tests may soon replace culture-based tests, necessitating fundamental changes in our public health efforts to track and control infectious diseases. Modernizing PulseNet to meet these challenges through new technologies and expanding laboratory capabilities is a priority for CDC. The need for enhanced capacity extends beyond PulseNet, however, to other national surveillance systems such as the National Antimicrobial Resistance Monitoring System. Fortunately, building the bioinformatics and laboratory infrastructure to support these new technologies for one surveillance system can serve as a platform for their use across other systems.

Laboratories play an important role in stopping contaminated food from reaching the public. Ensuring that PulseNet and other national infectious disease surveillance systems incorporate the molecular and bioinformatics technologies that are reshaping the practices of microbiology and infectious disease control is a critical priority for CDC and public health.

As the use of these next-generation sequencing technologies continues to expand, it is essential that CDC's national surveillance systems and laboratory infrastructure encompass the technology to ensure that infectious disease surveillance efforts are standardized and to enable CDC and its public health partners to most accurately detect, respond, and implement actions to contain newly emerging and well- recognized infectious threats regardless of their source.

Exhibit 405

American
Clinical Laboratory
Association

Statement

Of

Alan Mertz,
President,
The American Clinical
Laboratory Association

For

U.S. House of Representatives
Energy and Commerce Committee

Subcommittee on Health

Hearing on

21st Century Cures:
Examining the Regulation of Laboratory-Developed Tests

September 9, 2014
9:30 a.m.
2322 Rayburn House Office Building

American Clinical Laboratory Association
1100 New York Avenue, NW
Suite 725 West
Washington, DC 20001
202-637-9466
www.acla.com

Introduction

Chairman Pitts, Ranking Member Pallone, and members of the Subcommittee, the American Clinical Laboratory Association (ACLA) is pleased to have this opportunity to testify at today's hearing, "21st Century Cures: Examining the Regulation of Laboratory- Developed Tests."

ACLA is a not-for-profit association representing the nation's leading providers of clinical laboratory services, including local, regional, and national laboratories. Our diverse membership represents a broad array of clinical laboratories, includes large national independent labs, reference labs, esoteric labs, hospital labs, and nursing home laboratories. ACLA members are actively engaged in the creation and performance of innovative and much-needed Laboratory-Developed Tests (LDTs) that have helped to transform the standard of clinical care in this country and provide great hope for further improvements in the future.

ACLA and its member laboratories are committed to developing and providing safe, reliable, and clinically-meaningful diagnostic testing services to patients and ensuring adequate and appropriate regulatory oversight of the tests they perform. We do appreciate the willingness of the FDA to engage in a dialogue with our organization regarding its proposal, and the Agency has reached out to us. ACLA and its member laboratories are in the process of analyzing the documents released on July 31, 2014, and we fully intend to provide detailed and thoughtful comments on the documents once they are formally released as draft guidance. However, ACLA and the FDA fundamentally disagree on several key issues, including their statutory authority to regulate LDTs and the promulgation of new regulatory oversight through guidance documents,

and ACLA has other concerns related to the framework as outlined in the Congressional notification documents, all of which will be addressed in the following written statement.

In our testimony, we wish to highlight the following areas:

- The vital role and value of diagnostics and Laboratory-Developed Tests in clinical care;
- The current regulatory framework governing Laboratory-Developed Tests;
- The lack of statutory authority for the FDA to regulate Laboratory-Developed Tests;
- The FDA's Claim of jurisdiction over LDTs and its policy of "enforcement discretion" are relatively recent;
- The inappropriateness of the guidance process for regulating LDTs;
- Questions and concerns with FDA proposed framework;
- FDA's inadequate resources to handle the increased workflow;
- FDA regulation could severely affect patient access to cutting-edge diagnostics; and
- Effective modernization of current regulatory oversight to address new technologies and advancements

The Vital Role of Diagnostics, and LDTs, in Clinical Care

Laboratory-Developed Tests (LDTs) are tests that laboratories develop and validate in their own laboratories and that are not sold as kits to other laboratories or to other facilities. LDTs also include tests where laboratories modify an existing FDA-approved or FDA-cleared kit and then validate the modified test internally. LDTs are an extremely common part of laboratory medicine. Laboratory-Developed Tests are the backbone of clinical care in the United States.

The diagnostic information they yield empowers patients and their doctors with the tools they need to best manage patient care.

A large proportion of the clinical laboratory tests performed in this country are performed as LDTs, from routine tests such as pap smears and complete blood counts, to the most cutting-edge molecular and genetic tests in cancer, heart disease, and rare and infectious diseases. These are tests developed by physicians, scientists and other highly-trained personnel working in a single laboratory, according to its own processes, to furnish a diagnostic result for use by a clinician. These tests most often are created in response to an unmet clinical need, or where the existing diagnostic tests are insufficient or fail to incorporate the latest in scientific and medical research. Nearly all FDA-approved and FDA-cleared test kits begin as LDTs, and, in many cases, LDTs represent the standard of care.

Through the innovations in clinical laboratories, we are diagnosing and characterizing diseases earlier and more precisely than ever before imagined – whether for diabetes, infectious disease, cancers, and rare diseases. With these powerful diagnostic tools, patients have access to more targeted therapies sooner, which inevitably lowers costs, increases the quality of care, and saves lives.

Current Regulatory Framework Governing Laboratory-Developed Tests

The clinical laboratory industry has been extensively regulated for decades under a comprehensive, interlocking framework of federal laws, state laws, and peer review "deemed" authorities. The primary federal law governing labs has been the Clinical Laboratory Improvement Amendments (or CLIA), specifically the Clinical Laboratory Improvement

Amendments of 1988.[1] CLIA creates stringent requirements governing the operation of clinical laboratories to ensure the safe and accurate function of laboratories and the testing services they provide. These requirements cover the laboratories themselves, the necessary certifications for laboratory personnel from pathologists and geneticists to technicians, and the documentation of procedures for individual clinical laboratory tests. In addition, laboratories also are subject to inspections under both CLIA and state law. Further, moderate and highly complex laboratories, including all ACLA members, can choose to submit to additional oversight through deemed peer review authorities, such as the College of American Pathologists, the Joint Commission, and others, which add additional expertise in reviewing both the operation of the laboratory and the analytical and clinical validity of individual tests. This additional oversight for moderate and high complexity laboratories also involves the use of proficiency testing to ensure the accuracy of testing results. A group of 23 lab directors from the nation's leading academic medical centers wrote to the Acting Director of the Office of Management and Budget on July 16, 2014 and stated that "as part of this oversight, clinical laboratory physicians and scientists, including most of the signatories to [the] letter, perform careful inspections of laboratory facilities, exhaustive review of test protocols and validation, and continually monitor laboratory performance. This regulatory framework requires both extensive validation and continuous monitoring to ensure the performance, quality, and reliability of diagnostic services, yet allows laboratories the flexibility to develop and validate lab tests quickly and, thus, more quickly adopt new scientific knowledge and rapidly respond to unmet public health needs."[2]

[1] Pub. L. 100-578.

[2] http://www.aruplab.com/AboutARUP/PressRoom/PressRelease/2014/Letter-to-OMB-from-Lab-Leaders.pdf

Operating under this comprehensive yet flexible LDT oversight framework, the field of laboratory medicine has thrived, producing some of the most spectacular advances in medicine to occur in the last century. As highlighted in the aforementioned academic medical center lab director letter to OMB, "LDTs have long addressed emerging public health risks, such as HIV. For example, no HIV-1 antibodies confirmatory test was available when the HIV-1 screening test was introduced in 1985. Clinical laboratories developed and validated an LDT Western blot to meet the critical need to establish definitive diagnoses of HIV-1. It took two years before an FDA-approved Western blot test became available. Even now, the FDA-approved Western blot kit has not significantly changed since its first approval. Because obtaining additional FDA approvals for test kit modifications would be so burdensome, the manufacturer has not modified the test to keep up to date with the medical science."[3] Advances such as these "came about because of, and would not have been possible without, the current regulatory framework governing LDTs."[4]

LDTs have transformed clinical practice and dramatically altered treatment guidelines, as illustrated by the impact of Onco*type* Dx, a genomic LDT shown to predict whether chemotherapy is likely to benefit women with early-stage invasive breast cancer. Whereas 50 years ago, all women with breast cancer were referred for intensely toxic and debilitating chemotherapy treatments, we now know that only about 4 in 100 women diagnosed with early-stage breast cancer actually receive benefit from chemotherapy.[5] In the last ten years, the

[3] http://www.aruplab.com/AboutARUP/PressRoom/PressRelease/2014/Letter-to-OMB-from-Lab-Leaders.pdf

[4] *Id.*

[5] Paik S, Tang G, Shak S, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Oncol. 2006; 24(23): 3726-34.

Onco*type* Dx breast cancer test has helped over a hundred thousand patients around the world avoid chemotherapy and its side effects while saving the healthcare system an estimated more than $2.5 billion in treatment costs.

FDA Lacks the Statutory Authority to Regulate Laboratory-Developed Tests

As detailed in the Citizen Petition filed by ACLA last year, ACLA strongly believes that the FDA cannot regulate LDTs, through guidance or otherwise, because the Agency lacks the requisite statutory authority to regulate these vital diagnostic services.[6] FDA lacks the jurisdiction to regulate LDTs for several reasons.

LDTs are not "devices" as defined in the Food, Drug and Cosmetics Act (FDCA).[7] As the text and legislative history of the "device" definition show, this term encompasses only articles. LDTs are proprietary procedures for performing a diagnostic test using reagents and laboratory equipment. They are essentially know-how, not physical articles. Therefore, they are not subject to regulation under the FDCA.

Additionally, FDA's assertion of jurisdiction over LDTs is incompatible with the 1988 Amendment to the CLIA program (CLIA '88) and its legislative history. In amending CLIA, Congress explained its intent to regulate laboratory testing under a single statute: the amended CLIA. To that end, Congress created a comprehensive statutory framework for precisely the services that FDA now seeks to regulate under the device authorities of the FDCA. Congress

[6] ACLA Citizen Petition, Docket No. FDA-2013-P-0667 (Jun. 4, 2013), *available at* http://www.acla.com/wp-content/uploads/2013/12/060413-Citizen-Petition-to-FDA-Regarding-Laboratory-Developed-Tests-LDTs.pdf.

[7] 75 Fed. Reg. 34463 , 34463 (June 17, 2010).

made no mention of FDA having any authority to regulate LDTs under the previously enacted "device" definition.

Lastly, LDTs do not present an essential prerequisite for FDA jurisdiction under the FDCA: commercial distribution. FDA has defined "commercial distribution" in various contexts to require that a product be delivered, distributed, or placed on the market. LDTs are created and performed in a single laboratory, not manufactured and distributed. As non-tangible know-how and testing services at clinical laboratories, LDTs do not meet any of these conditions.[8]

The FDA's Claim of Jurisdiction over LDTs and its Policy of "Enforcement Discretion" are Relatively Recent

The FDA says that Congress gave the agency statutory authority to regulate LDTs nearly forty years ago when Congress passed the Medical Device Amendments of 1976 (MDA). The agency said that, since that time, it has opted to "exercise enforcement discretion" until now. That claim is contradicted by a review of actions and statements by Congress and the FDA throughout the years. It was not until twenty years after passage of the Medical Device Amendments that the FDA publicly stated that it could – but chose not to – regulate LDTs.

The legislative history of the Medical Device Amendments of 1976 contains no statement by the FDA or documentation submitted by the FDA to Congress that the agency considered LDTs to be "devices" under the framework of the MDA. Indeed, the legislative history shows that Congress itself believed that "devices" are tangible products and articles, but not processes such

[8] ACLA Citizen Petition at 2.

as LDTs.[9] Subsequent to passage of the MDA, when the agency undertook the rulemaking process and established advisory committees to classify all known devices, it did not mention then-existing LDTs as being "devices" subject to classification and regulation. If, in fact, the FDA thought at that time that LDTs were "devices" that it had the authority to regulate, then one would expect that the FDA would have explained to stakeholders why it was declining to classify them for regulation, but it did no such thing.

In 1988, Congress passed the Clinical Laboratory Improvement Amendments, which established a comprehensive statutory and regulatory framework for oversight of all clinical laboratory testing on humans in the United States. During the time that Congress was debating the legislation, the FDA stood by in silence, never once claiming that it had jurisdiction over any clinical laboratory tests developed in-house. The CLIA regulations that were finalized in 1992 did not include a regulatory role for the FDA with respect to LDTs or any other lab processes, and we are not aware that the FDA sought to assert such a role at the time.

The first time that the FDA made a public claim about its authority to regulate LDTs as devices was in a draft guidance document in 1992.[10] Stakeholders objected, and the FDA removed any reference to LDTs in the final guidance, released in 1996.[11]

It was not until 1996 – two decades after the Medical Device Amendments – when the FDA claimed in a statement in an official publication, the Federal Register, that it had jurisdiction

[9] S. Rep. No. 94-33, 94th Cong., 1st Sess. at 17 (Mar. 11, 1975).

[10] Draft Compliance Policy Guidance: Commercialization of Unapproved In Vitro Diagnostic Devices Labeled for Research and Investigation (undated) at 4.

[11] *See* FDA, Compliance Policy Guide 7124.32, Commercialization of In Vitro Diagnostic Devices (IVDs) Labeled for Research Use Only and Investigational Use Only (May 1996).

over LDTs but that it was not exercising its authority to regulate them. It hinted at its jurisdictional authority and its exercise of enforcement discretion, stating that although it had not "actively regulated" LDTs, it might do so in the future.[12] At the time, ACLA and other stakeholders filed comments challenging the FDA's assertion that it had the authority to oversee LDTs for twenty years but simply never used that authority. In 1998, in its denial of a citizen petition on LDTs, the FDA again stated that it "may regulate assays developed by clinical reference laboratories strictly for in-house use as medical devices."[13] This assertion has been repeated in the years since then, although it was not until recently that FDA determined that it would use its purported enforcement authority for the first time.

The Inappropriateness of the Guidance Process for Regulating LDTs

The FDA takes the position that it has the jurisdiction to regulate LDTs but has always chosen to exercise its regulatory discretion with regard to those tests. The clearest statement of that discretion is found in the FDA's announcement of the Final Rule regulating Analyte Specific Reagents, which are the component of many LDTs. In promulgating the ASR Rule, the FDA declined to classify Laboratory-Developed Tests as Class II or III medical devices because, as the agency stated, "FDA recognizes that the use of in-house developed tests has contributed to enhanced standards of medical care in many circumstances and that significant regulatory changes

[12] Medical Devices; Classification/Reclassification; Restricted Devices; Analyte Specific Reagents, 61 Fed. Reg. 10484 (Mar. 14, 1996).

[13] FDA Response to Hyman Phelps & McNamara, P.C., Citizen Petition, Docket No. 92P-0405 (Aug. 12, 1998).

in this area could have negative effects on the public health."[14] In announcing a change to that policy, FDA cannot proceed simply through the issuance of guidance documents.

First, given that the original announcement of this policy was as part of a notice and comment rulemaking, the reversal of the policy—which FDA is asserting here—must be done in the same way. Because FDA set forth its policy regarding Laboratory-Developed Tests in the Federal Register, pursuant to notice-and-comment procedures, if the agency is going to change its policy, then it must follow that same notice-and-comment procedure.[15]

There is little question that by its actions, FDA is expanding its current regulations to an entirely new industry. The FDA cannot newly regulate an entire industry sector merely by issuing a few guidance documents. Federal courts long have held that when a guidance document significantly broadens the application of a regulation or set of regulations, it is invalid without actual notice-and-comment rulemaking.[16] It is also well-established that an agency cannot sidestep notice-and-comment rulemaking requirements by claiming that a major legal addition to a rule is merely an interpretation of an existing obligation.[17] Here, if the FDA's guidance is in any way similar to the documents the FDA shared with Congress in July, it would expand the application of existing regulations that currently are not applicable to laboratories offering LDTs. In some cases, the guidance would completely contradict what is in current regulation, which in itself would require notice-and-comment rulemaking. Expansion of the FDA's regulatory regime to

[14] 62 Fed Reg. 62243, 62249 (Nov. 21, 1997).

[15] *See, e.g., Ball Memorial Hospital v. Leavitt*, 2006 WL 2714920 (D.D.C. 2006).

[16] *See, e.g., Appalachian Power Co. v. EPA*, 208 F.3d 1015 (D.C. Cir. 2000).

[17] *See Paralyzed Veterans of America v. D.C. Arena L.P.*, 117 F.3d 579 (D.C. Cir. 1997).

LDTs significantly broadens the scope of current regulations to an entire industry, and it would be far more than an interpretation of an existing obligation on labs. Therefore, according to years and years of federal court rulings, the FDA cannot regulate LDTs through subregulatory guidance documents alone.

Furthermore, the FDA cannot claim, as it often does with regard to guidances, that these documents "do not establish legally enforceable responsibilities" and that they merely "describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited." It includes such language in all of its guidance documents, including those it shared with Congress in July. But, if finalized, the LDT guidance documents most certainly would impose legally enforceable responsibilities on labs, and they contain far more than just "recommendations." The documents we have seen are packed with citations to specific existing statutory and regulatory provisions and very direct statements that LDTs for the first time would be subject to those provisions. As an example, the FDA states that any lab that fails to follow certain other requirements in the document "will have opted to not be within the scope" of the FDA's current policy under which labs do not have to register and list their tests.[18] If device registration and listing is not a "legally enforceable responsibility" that suddenly would be imposed on labs, then it is hard to see what would be. There are many other examples of legally enforceable responsibilities on virtually every page of the documents the FDA shared with Congress that completely contradict the agency's claim that the guidance is just describing its current thinking and making recommendations.

[18] Anticipated Details of the Draft Guidance for Industry, Food and Drug Administration Staff, and Clinical Laboratories, Framework for Regulatory Oversight of Laboratory-Developed Tests (LDTs) at 17.

Moreover, though the Agency stated that in its July 31, 2014 response to the ACLA Citizen Petition [attached] that "any such guidance would not establish any legal obligations" under the theory that the legal obligations arise under the FDCA itself, this is plainly not true.[19] As summarized to Congress, the final guidance would clearly obligate laboratories, under threat of enforcement action to newly comply with FDA regulations and guidances. Some of these obligations are the same as seen by device manufacturers, but others are completely novel and not grounded in any statute or regulation.[20]

The difference between proceeding through guidance and proceeding through regulation is not merely an academic one. The FDA's "Good Guidance Practices" do not extend the same rights and protections to all stakeholders that notice-and-comment rulemaking would.[21] There are key differences in the obligations imposed upon the FDA – or any federal agency – when engaging in rulemaking, versus the requirements the FDA follows with respect to guidance. Although the FDA plans to accept public comment on the draft guidance, unlike notice-and-comment rulemaking, the FDA is not required to respond to stakeholder comments and explain its rationale for amending draft guidance – or not.[22] This is critically important to understanding the "agency's current thinking." The FDA is also not required to conduct any burden analysis or regulatory impact analysis when it issues guidance, both of which are standard features of notice-and-comment rulemaking. If the agency did proceed through notice-and-comment rulemaking, there is no doubt

[19] FDA Response to ACLA Citizen Petition, Docket No. FDA-2013-P-0667 (Jul. 31, 2014).at 15.

[20] *See*, e.g., Anticipated Details of the Draft Guidance at 16. The FDA plans to require laboratories to submit "notification" of basic information about LDTs to the Agency, yet no such framework exists in statute or regulations for other "device" manufacturers.

[21] Food and Drug Administration Modernization Act of 1997, Pub. L. 105-115 (1997), § 405; 65 Fed. Reg. 56468 (Sept. 19, 2000).

[22] *See* 21 C.F.R. § 10.115(g)(iv).

that it would have to put the public on notice that its plans to start regulating an entire industry sector are likely to have a major impact on the entire laboratory industry.

ACLA strongly opposes the claim that the FDA has the authority to regulate Laboratory-Developed Tests. However, if the agency nevertheless moves forward in its attempt to regulate LDTs, it most certainly cannot do so merely through guidance documents. It must use notice-and-comment rulemaking to vastly expand the application of existing regulation and to amend those regulations that do not apply to LDTs or that contradict its plans for regulating LDTs.

FDA's Guidance Documents Raise Real Concerns Due to Unanswered Questions

The documents released by the Agency on July 31, 2014 go far beyond reflecting current Agency thinking, as they propose an entirely new regulatory framework that will be applied to clinical laboratories developing LDTs for the first time. If the FDA were to finalize this guidance, it would represent nothing short of a wholesale reimagining of the regulation of laboratories, subjecting laboratories to an entirely new set of requirements that they have never faced before.

The Agency has put forth a high-level, conceptual vision of how it would regulate LDTs, while providing very little concrete guidance to the laboratories as to what specifically the FDA will require and how to devise a compliance strategy or operationalize the requirements.

Interplay of FDA Requirements with Existing LDT Oversight Under CLIA

There is no discussion of how any additional regulation by the FDA would interact with the regulation already in place under the CLIA program, including those functions performed by deemed authorities. There are many areas of commonality and overlap, specifically with respect to validation, inspections, and quality systems regulation, and yet there is no discussion of how

two separate regulatory authorities would regulate the laboratory industry in a way that would not impede innovation. The Agency had discussed a third guidance document that it planned to release with the actual draft guidance, a document which was to specifically address how the Quality Systems Regulation (QSR) requirements applicable to devices under the FDA would interplay with the quality requirements under CLIA.[23] The Agency has stated that it no longer plans to release such a document with the actual guidance documents. Rather, it has said it will rely on a third-party organization to explain how CLIA and FDA's QSR requirements can be reconciled. ACLA believes it is wholly inappropriate for FDA to leave such a vital issue to an unaccountable third party to resolve.

What Is the "Device" to be Regulated, and Where Does "Manufacture" Take Place vs. Test Performance

The documents released by the FDA fail to address the fundamental differences between device manufacturers and clinical laboratories. Unlike manufacturers of IVD test kits, laboratories are both the innovators and providers of clinical laboratory services, utilizing their advanced knowledge, training, and education in the practice of laboratory medicine to deliver the highest quality health care services for millions of real, every day patients. Knowing this, it would be unreasonable to deem a laboratory, "a manufacturer" and claim that there is a "level playing field," when manufacturers and laboratories run fundamentally different operations.

[23] *See, e.g.*, Minutes from Negotiation Meeting on MDUFA III Reauthorization: June 27, 2011, at 3, *available at* http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/Overview/MedicalDeviceUserFeeandModernizationActMDUFMA/ucm263026.htm.

Unlike a device manufacturer, which produces a test kit or device that then is sold to another entity that ultimately performs the test, a clinical laboratory is an integrated operation consisting of highly trained and certified personnel who design, validate, perform, and interpret laboratory tests to furnish test reports that then can be used by ordering physicians, in concert with other information, to make treatment decisions. Defining exactly what the "device" is that FDA seeks to regulate, or where the "manufacture" of the test ends and the performance of the test begins, has yet to be explained.

What are "High Risk" and "Moderate Risk" LDTs?

Under the proposed regulatory framework described in the documents released on July 31, 2014, the FDA will not issue draft guidance describing the risk classification of LDTs for 18 months after the finalization of the guidance, with final guidance on risk classification not being issued for two years after the finalization of the guidance. The Agency and stakeholders have spent years attempting to define "high risk" and "moderate risk" in the context of clinical diagnostics, and it is crucial that the Agency clearly define such fundamental principles before instituting a new regulatory framework based on those definitions.

Defining "Adverse Events" and "Device Malfunctions" In the Context of LDTs

It is unclear in the context of LDTs what constitutes an "adverse event" that must be reported by a laboratory. For example, how precisely would a laboratory test contribute to the death of, or serious injury to, a patient? Would the FDA consider it an "adverse event" if a patient's cancer returned after an LDT test predicted a 90 percent chance that cancer would not return? Even if "adverse events" were defined in a way that applied in the diagnostic context, it is not clear from an operational standpoint how laboratories could be expected to report adverse

events. Referring physicians use LDT test results as one part of a broader clinical picture to make treatment decisions for patients, and these clinical decisions and patient encounters often occur outside the laboratories' knowledge or involvement. Thus, laboratories would not have access to information on a patient's other clinical inputs or prognosis after the test results are reported to referring physicians.

Similarly, it is unclear in the context of LDTs what constitutes a "device malfunction" that the LDT "manufacturer" would be required to report to the FDA under 21 C.F.R. § 830.50(a). This issue arises in part because the FDA is seeking to regulate a service rather than a product, and in part because of the FDA's expansive view of the test system as including, for example, patient demographics, sample procurement and preparation, and reporting. Would an error in patient demographic data entry constitute a "device malfunction" if it had no effect on the test result? What if a momentary interruption in result reporting were to occur due to information system technical difficulties, but the problem was promptly resolved without significantly affecting the timeliness of result delivery? If broadly interpreted and enforced, the requirement to report "device malfunctions" could overwhelm laboratories with reporting incidents that have no adverse effect on the test results or patient care.

Modifications to FDA-Approved and Cleared Tests

High complexity clinical laboratories frequently purchase FDA-approved or FDA-cleared test kits from device manufacturers and modify these test kits, thereby creating LDTs, to improve the performance of the diagnostics, address problems or issues with the FDA-approved or cleared devices, or to incorporate the latest research and clinical knowledge. For instance, a well-known FDA-approved ALK gene FISH test kit, an *in vitro* companion diagnostic used to

aid in treatment selection for patients with Non-Small Cell Lung Cancer, was found by one lab to suffer from poor assay performance. These tests, as LDTs, current are regulated by CLIA and undergo the necessary validations as outlined earlier in this document.

The Agency has stated in the framework documents released to Congress on July 31, 2014 that any modifications to "an FDA cleared/approved device in a way that affects device performance or intended use is considered to be a device manufacturer… [and] [t]hese modified devices must meet premarket submission requirements."[24] To force a laboratory to undergo such a burdensome and expensive premarket review process in order to make modifications to an FDA-approved or cleared test kit is unreasonable, an encroachment on the practice of medicine, and will be a disincentive for laboratories that otherwise would make such changes to improve diagnostic capabilities of FDA-approved or FDA-cleared tests, which will negatively impact patient access to cutting edge diagnostics.

Are anatomic pathology services considered LDTs subject to FDA regulation?

The anticipated details of this draft guidance leave unclear the regulatory status of many anatomic pathology services provided by laboratories. Anatomic pathology services typically involve the preparation of a biopsy or cellular specimen on a slide (the "technical component") for microscopic examination and interpretation by a pathologist (the "professional component"). Examples of such services include histopathology or surgical pathology, cytopathology (including the Pap smear test), and hematology. These procedures may include FDA-approved or -cleared components and instruments, components that are exempt from FDA premarket

[24] Anticipated Details of the Draft Guidance at 26.

review, or modifications of FDA-approved, -cleared or -exempt components or instruments, and are often performed in laboratories that are independent of health care facility laboratories.

It is difficult to see how the FDA could consider a pathologist reviewing a slide as an *in vitro* diagnostic or an LDT; in this instance, the pathologist is practicing his or her field of medicine just as any other physician when practicing medicine in his or her office. However, the Agency has written the anticipated details of the draft guidance so broadly that they appear to sweep into the risk-based framework any procedure a laboratory performs that is intended for clinical use and is not an unmodified FDA-approved or -cleared test kit, unless specifically excepted. Under what circumstances, if any, would the FDA view the technical component, the professional component, or the technical and professional components of anatomic pathology together, as a "test system" constituting an LDT subject to the risk-based framework?

FDA Lacks the Resources to Handle the Increased Workflow

We also have very real concerns about resource constraints within the Agency to effectively manage this entirely new area of diagnostic regulation. There are tens of thousands of LDTs in existence today, with hundreds of new tests created every year.

According to CMS, of the 36,432 non-waived laboratories regulated under CLIA, 11,633 CLIA certified laboratories perform at least one or more specialties categorized as high-complexity, which is the only category of labs that are permitted to perform LDTs. A majority of these 11,633 laboratories develop and perform LDTs, many of which could be classified as moderate- or high-risk, depending upon how FDA tailors the risk classifications two years after the finalization of the framework guidance.

In 2013, the FDA approved 23 pre-market approval applications.[25] The Agency has stated in calls with industry stakeholders that it anticipates that the initial set of submissions for the "highest risk" LDTs will be around 100 tests, a number we believe falls far short of the actual number. This is an incredible workload for any agency or organization to undertake, and ACLA has serious concerns about the FDA's ability to handle this additional workload.

FDA Regulation Could Severely Affect Patient Access to Cutting-Edge Diagnostics

Subjecting LDTs to FDA regulation would eliminate the very characteristics which makes LDTs and the regulatory framework that presently govern them so vital: flexibility and nimbleness in their ability to respond to unmet needs. The flexibility afforded under the CLIA regulatory framework allows laboratories to develop tests quickly and to update them regularly as research and medicine advances, giving patients access to the most current diagnostic testing available. Such flexibility would be lost under the FDA device regulatory framework.

Additionally, FDA regulation of LDTs as medical devices would dramatically slow not only the initial premarket approval of new tests, but also improvements to existing tests, delaying access to new and improved diagnostic testing services for patients and clinicians. Under the current CLIA regulatory framework, laboratories may continually modify and update their tests to reflect medical research advances, provided that the laboratory appropriate validate and document test modifications. Under the FDA device regulatory framework, and as outlined in the proposed LDT framework provided to Congress on July 31, 2014, these modifications would

[25] *See, e.g.* *http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/DeviceApprovalsandClearances/PMAApprovals/ucm344734.htm*.

require supplemental filings and authorizations from the FDA.[26] These additional authorizations can take months to obtain, and in many cases, laboratories could not implement the modifications in the interim. Therefore, FDA regulation would impede scientific progress in clinical diagnostics.

ACLA Has Supported Modernization of Current Regulatory Oversight to Address New Technologies and Advancements

As ACLA stated in its June 2013 Citizen Petition to the FDA, "The CLIA framework has worked very well. Over the past few decades, health care providers have ordered millions of LDTs for their patients with few problems. With regard to genetic tests, for example, the Secretary's Advisory Committee on Genetics, Health, and Society has stated that 'there have been few documented cases in which patients experienced harm because of errors in a CLIA-regulated genetic test.'[27] Even though laboratories are not required to report adverse events, litigation or other publicity likely would have revealed more widespread incidence of harm if such harm had in fact occurred. Thus, regulation of LDTs under CLIA has effectively protected the public health.

To the extent that stakeholders have concerns about possible gaps in the clinical validation of LDTs, the most logical and appropriate solution would be to amend CLIA and/or its regulations. It would be overly burdensome to superimpose a new bureaucratic regime on the laboratory industry which is already highly regulated under CLIA. It also would be like trying to

[26] *See, e.g.,* FDCA § 515(d)(6), 21 C.F.R. §§ 807.81(a)(3), 814.39; Anticipated Details of the Draft Guidance.

[27] Secretary's Advisory Committee on Genetics, Health, and Society, "U.S. System of Oversight of Genetic Testing: A Response to the Charge of the Secretary of Health and Human Services" (Apr. 2008), at 32, *available at* http://oba.od.nih.gov/sacghs/sacghs_oversight)report.pdf.

fit a square peg into a round hole to impose an additional layer of regulation based on a statute designed for products (FDCA) rather than laboratory testing procedures."[28]

ACLA and its member laboratories have always been committed to ensuring patient access to accurate, reliable, and meaningful clinical laboratory tests that improve the quality of care, decrease costs, and improve the lives of patients. ACLA has long supported modernizing the regulatory requirements under the CLIA program to keep pace with changing technology. We are confident there are policies that can be developed to accomplish this without doubling or tripling the regulation, oversight and cost.

Conclusion

Chairman Pitts, Ranking Member Pallone, and members of the Subcommittee, thank you for this opportunity to testify today. ACLA is grateful for the opportunity to share our view on the regulation of Laboratory-Developed Tests. The Path to 21st Century Cures Initiative has shown that medical innovation in the U.S. has moved health care ahead by leaps and bounds and even more exciting innovations are just on the horizon. The Initiative has also shown that clinical laboratory diagnostics are a critical and powerful tool in this effort and will enable us to provide patients with higher quality health care at lower costs. To the extent that additional oversight of LDTs is necessary, we continue to believe that the best vehicle for that is modification of CLIA, which already extensively regulates LDTs. ACLA commends you for your leadership and looks forward to working with you, the FDA, and the Administration to

[28] ACLA Citizen Petition at 18.

ensure regulation of LDTs strikes the right balance between innovation, safety, and patient access.

Exhibit 406

Medical Laboratories

Lab Handbook & Test Directory

General Information

Introduction

General Laboratory Policies

Lab Requisitions

Lab Specific Information

Autopsy Service

Blood Bank and Transfusion Medicine Service

Clinical Core Laboratory

Cytogenetic Laboratory

Cytology Laboratory

Davis Laboratory

Emily Couric Clinical Cancer Center Laboratory

Fontaine Laboratory Services

Microbiology and Molecular Diagnostics - Infectious Diseases

Faculty Director:	Melinda Poulter, Ph.D
Associate Director:	Amy Mathers, MD
Scientific Director:	Larry Silverman, Ph.D, D (ABCC), (ABGM), F.A.C.M.G.
Manager:	Michael Sidlak, MS, SM(ASCP)CM
Technical Specialist:	
TELEPHONE:	434-982-0460
DIRECTOR-ON-CALL:	PIC 1221
HOURS:	Open 24 hours/day

General Guidelines and Policies for Microbiology

The most important contribution to the effectiveness of the microbiology laboratory is the **specimen** that is **appropriately selected, collected and transported**. Since specimens for microbiological analysis are likely to contain living organisms, specimen collection, handling and transport should be accomplished with the following factors in mind:

1. Select the **correct anatomic site** from which to obtain the specimen and **communicate** this to the laboratory via the appropriate request mechanism (see Section I General Policies).

2. Collect the specimen using the **proper technique and supplies** (refer to the individual test listings for this information).

3. Package the specimen in a **container designed to promote survival of the causative organism and to eliminate leakage**. **Label completely** (refer to Section I General Policies B).

4. Transport the specimen to the laboratory **expeditiously** or make sure

Histocompa Laboratory
Medical Laboratorie Administrat
Microbiolc and Molecular Diagnostic - Infectious Diseases
Molecular Diagnostics / Human Genetics
Specimen Collection / Phlebotomy
Specimen Managemer and Referral Testing Services
Student Health Laboratory Services
Surgical Pathology Laboratorie
Toxicology
Appendices
Clinical Pathology
Current Antimicrobial Susceptibility Profiles
Cytogenetics
Cytology
Surgical Pathology
Appendices
Search for Lab Tests
Lab Requisition Forms

that, if it must be stored, the storage conditions are appropriate for the suspected organism.

It is also important to note that the laboratory needs specific and critical information from the physician regarding the patient and the specimen. Please refer to Section I for detailed information about requesting laboratory analysis. If a particular agent/organism is suspected, please communicate this fact to the laboratory when making requests for analysis.

If a specimen is determined unacceptable for culture, it will be retained until the requesting physician is notified.

The report of "**No Significant Change**" indicates that the same organism/s was isolated from the same site within the last 72 hours. After 72 hours, cultures submitted from the same site will be repeated with full identification and susceptibility if appropriate. **The laboratory will evaluate culture results and perform identification and susceptibility on potential pathogens for the clinical site. An additional charge will be generated for each identification and susceptibility performed. Reports will be updated daily via the laboratory and hospital computer system.**

Bacteriology Specimen Submission Guidelines

Anaerobic- cultures will be performed on the following specimens when the specimens are submitted in an approved transport container, appropriately labeled and ordered, and meet the requirements for anaerobic culture (an additional charge will be assessed for anaerobic culture):

Aspirates

Sterile Body Fluids

Bone Tissues

Tissue/Biopsy

Specimens for anaerobic culture require special collection and transport techniques to prevent loss of anaerobic conditions. Culturettes are unacceptable for anaerobic cultures. Aspirates or biopsies are the preferred specimens. Sterile body fluids should be submitted in a sterile cup or a sterile black top tube. Sterile fluids should not be submitted in blood culture bottles because no gram stain or quantification of organisms can be made. Place tissues in a sterile cup. Tissue specimens should be no larger than marble size. All specimens for anaerobic culture should be submitted to the laboratory within one hour after collection.

Samples from superficial sites or sites that have anaerobic normal flora are not suitable for anaerobic culture.

Lab Compliance

Laboratory Medicine Updates

Clinical Trials Program

Directory of Medical Laboratory Personnel

Intranet

Aerobic (but not anaerobic) cultures are performed on virtually any body site provided the specimen is submitted in an approved transport container, appropriately labeled and ordered. See individual test listings for specific specimen requirements. All swabs should be submitted using the Culturette II system available from the hospital storeroom. Blood Cultures: A two bottle CO_2 detection system is employed for the recovery of aerobic and anaerobic bacteria and yeast. Bottles are available on nursing units or from the hospital storeroom. A peptide nucleic acid fluorescent in situ hybridization test (PNA FISH) will be used within several hours on smears from positive blood cultures. The test will detect *Staph aureus, Enterococcus faecalis* or *Candida albicans* within several hours of the positive blood culture detection. If routine methods of culture fail to provide a microbiological diagnosis, the physician is encouraged to consult with the senior microbiology staff. If unusual organisms are suspected, the laboratory must be notified by phone and the suspected agent should be noted on the requisition

Reporting of Results: All inpatient results will be reported through the hospital information system. Preliminary reports will be available by computer immediately after the completion of a screening test or the first reading. All cultures, as appropriate, are evaluated each day. Reports will be updated daily or whenever new information becomes available. The physician, or if appropriate, the chief resident of the service will be notified by telephone of positive blood, tissue, and sterile body fluid cultures as well as reportable and/or communicable disease isolates.

Refer to appendix VII for definitions of reports.

Antimicrobial Susceptibility Testing and Reporting: Automated Minimum Inhibitory Concentrations (MIC) results are reported in ug/mL with interpretations and are performed routinely on most clinically significant organisms.

S,I,R assignments are not possible for some drug / microbe combinations due to insufficient validation data. The term "non-susceptible" will be used in some cases.

Standardized disk diffusion tests are performed on some isolates and are reported as:

Susceptible: The isolate appears to be susceptible to ordinarily achievable blood levels.

Intermediate: Susceptibility of the isolate is indeterminate; some strains may respond to concentrations achieved by high dosage or in areas of the body where the drug is concentrated (e.g.urine).

Resistant: The isolate is not completely inhibited by drug concentrations within the usual therapeutic range.

Antibiotics which are tested, reported, and suppressed are determined by changing susceptibility within the health system, formulary changes, and new pharmacokinetic and pharmacodymanics data. These decisions are discussed annually with members of the Antimicrobial Stewardship Team and reviewed at AUC.

The Health System's antibiogram is created annually and posted on the Clinical Portal in the Laboratory Handbook.

Mycobacteriology Specimen Submission Guidelines

GUIDELINES FOR OBTAINING SPECIMENS

For cases of suspected pulmonary tuberculosis:

See test listing for Culture, AFB for optimal specimen types and volumes.

For outpatients, the three sputa can be collected at home in sterile containers, refrigerated, and all brought to the laboratory the same day.

Only 1 sputum and/or bronchoscopy specimen is accepted per 8 hours; three sputa and/or bronchoscopy specimens are accepted per week. If the smears from these specimens are negative, another set of three specimens is accepted after one week if the suspicion of tuberculosis remains.

Once the diagnosis of tuberculosis is established from the initial series of three sputa, only one sputum is accepted every other week for AFB smear. An AFB culture is done from an acceptable sputum every other week. **If more than one sputum is received by the laboratory within a week, the physician will be called to determine if the additional sputa are being submitted to discontinue isolation.** Exception: to discontinue isolation requires 3 consecutive negative smears from sputa obtained at least 8 hours apart.

Three consecutive negative (AFB smears) obtained 8 hours apart are required for release from isolation for Tuberculosis.

For cases of suspected non-pulmonary tuberculosis:

Swabs are not an appropriate collection device for most specimens submitted for mycobacterial culture. If swabs must be submitted, however, a separate swab must be included for the AFB culture. The clinician should be aware that the sample on a swab is suboptimal for the isolation of a mycobacteria species. Swabs will only be accepted from caseous lymph nodes, bone lesions, brain lesions, organ lesions, and other deep tissue lesions.

For urogenital tuberculosis, collect 3 early-morning, voided, midstream or catheter urines on successive days. For outpatients, the three urines can

be collected at home in sterile containers, refrigerated, and all brought to the laboratory the same day.

All other specimens are collected as you would for a bacterial culture.

Identification/ Reporting:

Molecular probes are used to identify *M. tuberculosis, M. avium,* and *M. gordonae.* Because the probe used for *M. tuberulosis* cannot distinguish between *M. tuberculosis, M. bovis, BCG, M. African, M. microti,* and *M. canetti*, a positive result is reported as *M. tuberculosis* complex.

The probe for *M. avium* cannot distinguish between *M. avium*, and *M. intracellulare*, therefore a positive result is reported as *M. avium* complex.

Mycobacteria not identified by probe are usually identified by 16S ribosomal DNA sequencing with results available within one to two weeks.

Susceptibility Testing:

M. tuberculosis complex is tested against isoniazid, streptomycin, ethambutol, rifampin and pyrazinamide. Between 1999 and 2006, 43 *M. tuberculosis* complex patient isolates were tested for susceptibility to INH. 6.9% of the isolates were INH resistant.

Mycology Specimen Submission Guidelines

Specimen requirements

See test listing Culture, Fungus

Suspicion of infection with the systemic fungal pathogens, *Histoplasma capsulatum, Blastomyces dermatitidis,* or *Coccidioides immitis* should be indicated on the test request.

If *Malassezia species* is suspected, please note this on the test request because the sample may require additional processing for recovery.

Susceptibility Testing

Candida species can be tested in house for susceptibility to fluconazole. Additional susceptibilities and susceptibility testing on Nocardia and mold species requires consultation with a laboratory technologist. These tests are performed by an accredited referral laboratory.

Virology Specimen Submission Guidelines

Culture and Direct Antigen Detection:

See individual test listings for Virus Cultures

Transport Media:

Viral transport medium (VTM) is available in the Clinical Microbiology Laboratory. VTM should not directly contact patients. Very acidic (yellow) or alkaline (purple) media should not be used. Observe the expiration date on the label. **The medium can not be used for bacterial culture specimens as it contains antibiotics.**

Specimen Collection for virus isolation:

The collection of a proper specimen and its correct handling are critical steps in virus isolation. Specimens for virus isolation must be collected and processed in a different manner than those for routine microbiologic studies. The following guidelines should be followed in submitting specimens for virus isolation:

1. Only two specimens for viral culture from the same site will be accepted within a seven day period per patient. **These two specimens must be at least 48 hours apart.**

2. The amount of virus is usually maximal at or just after the appearance of symptoms, so that specimens should be collected as early as possible in the course of illness.

3. Most viruses are inactivated by adverse environmental conditions and/or delays in specimen processing.

a. Whenever possible, specimens for isolation should be submitted fresh in the morning. Specimens should be transported to the laboratory immediately after collection (keep all specimens except blood refrigerated until transport). Blood should be kept at room temperature.

b. For unavoidable delays, specimens may be held at $4^{o}C$ (refrigerator) overnight, but they should not be frozen.

c. Fluctuations in temperature, especially freezing and thawing, should be avoided. Certain viruses, such as respiratory syncytial, cytomegalovirus, and varicella-zoster virus, are particularly labile, and specimens suspected of containing these agents should be collected at times when they can be processed promptly without freezing.

d. Containers for virus transport should be made of glass or plastic and have airtight lids.

e. Swabs and other samples that could dry out in transport should be placed in vials containing viral transport medium (VTM). Dry swabs are not acceptable specimens. **Culturette II swabs are not acceptable specimen systems for recovery of viruses.**

f. Only cotton or dacron swabs with aluminum or plastic shafts should be used for collection. Calcium alginate swabs are not acceptable for collecting virus samples nor are swabs with wooden shafts.

4. Bacterial overgrowth can seriously hinder efforts at virus isolation. Specimens should be collected aseptically with attention to minimize contamination by microbial flora and should be processed promptly.

5. When a virus is initially isolated, from blood or sterile body fluids or from known immunocompromised patients, the physician will be notified by telephone.

Susceptibility Testing:

Susceptibility testing on isolates of CMV, HSV and VZV is performed by special request at an accredited referral laboratory.

Viral Serology:

Serological testing for viral antibodies should be carried out on acute and convalescent sera. Individual tests are listed in the index. Questions concerning testing for viral antibodies should be directed to the Clinical Immunology (Davis) Laboratory (924-5179).

SITE OF INFECTION	COMMON VIRAL ETIOLOGIES[a]	APPROPRIATE CLINICAL SPECIMEN	BIOPSY/ AUTOPSY
Respiratory	Influenza A, B Parainfluenza Respiratory syncytial Adenovirus Picornaviruses	Nasal Washings Nasopharyngeal swab Throat swab Sputum Feces (if enteroviruses suspected)	Lung Bronchial scrapings or biopsy
Central Nervous System	Enteroviruses Herpes Simplex Adenovirus	CSF Throat swab Feces Urine (if mumps suspected)	Brain Parotid

Systemic or Congenital	Adenovirus Cytomegalovirus Enteroviruses Herpes Simplex	Feces CSF Urine Anticoagulated (EDTA) blood (purple top tube) Saliva (if CMV suspected) Sputum (if CMV suspected) Throat swab (except CMV)	Kidney Liver Lung Brain Heart Intestinal contents
Cardiovascular	Enteroviruses	Throat swab Feces Pericardial fluid	Pericardium Myocardium
Ocular	Adenovirus Herpes Simplex Enterovirus	Eye swab Throat swab	Conjunctival scrapings
Cutaneous, Vesicular or Ulcerative	Herpes Simplex Varicella-Zoster	Vesicle fluid Scrapings from Vesicle base Throat swab Feces (if enteroviruses suspected)	Liver Spleen Lung Brain
Cutaneous, Maculopapular	Enteroviruses Adenovirus	Feces Anticoagulated (EDTA) blood (purple top	Liver Spleen Kidney

		tube) Conjunctival swab	
Gastrointestinal	Rotavirus	Stool	

[a]Viruses which cannot be detected in our laboratory are not listed.

Molecular Diagnositic Submission Guidelines

Molecular testing is available for several infectious diseases including:

Herpes Simplex Virus types I and II

Enterovirus

Bordetella pertussis

C. difficile

Methcillin Resistant Staph aureus

Influenza A & B

HIV viral load and genotyping

HCV viral load and genotyping

Cytomegalovirus viral load

HBV viral load

BKV viral load

EBV viral load

Respiratory Viral Panel

Chlamydia trachomatis

Neisseria gonorrhoeae

16 S ribosomal sequencing for various bacterial and fungal isolates

See individual test listing for the particular organism of interest.

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Exhibit 407

UNIVERSITY OF WASHINGTON DEPARTMENT OF LABORATORY MEDICINE
Molecular Diagnosis Microbiology Section

Menu

Available Tests
Submitting a Specimen
Order Form (PDF)
Acceptable Specimens
Clinical Significance
Contact Us
Publications/Links
About Us
Staff Only

Questions?
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About Next Generation 16S Sequencing

Many sites of the human body are colonized by complex communities of microbes (the "human microbiome") in both health and various disease states. Highly diverse, polymicrobial specimens are often difficult, or even impossible, to fully characterize by techniques in common clinical use:

- Culture-based identification relies upon the ability of organisms to grow and replicate in vitro. Therefore, detection of fastidious or slow-growing organisms, or those rendered inviable due to processing (such as in formalin-fixed paraffin embedded tissue specimens) or during storage (such as anaerobes which have been exposed to oxygen) is limited. Furthermore, only a limited number of species can be practically classified by this approach.
- Molecular methods, such as our 16S rRNA gene sequencing assay, do not require prior culture and have improved ability to identify inviable or fastidious organisms from direct specimens. However, in a polymicrobial speciemen only the predominant organism may be identified, or an uninterpretable signal may be generated (**Figure 1**).

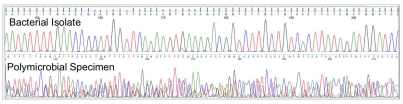

Figure 1. Examples of conventional 16S rRNA gene sequencing results from a bacterial isolate and a polymicrobial specimen. For the bacterial isolate (top), Sanger sequence data produces a clean electropherogram that can be used to provide a species-level taxonomic classification. For the polymicrobial sample (bottom), Sanger sequencing generates a different electropherogram for each species present, resulting in mixed signal which is uninterpretable.

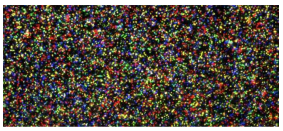

Figure 2. High-powered magnification of a next-generation sequencing run. Each fluorescent spot represents an individual DNA molecule undergoing sequencing. The color of the spot indicates the identity of the nucleotide being interrogated during the current sequencing cycle. Image from Shendure, Porreca et al. Science (2005).

In contrast to conventional approaches, next-generation DNA sequencing (alternatively termed "NGS", "high-throughput sequencing", "massively parallel sequencing", or "deep sequencing") provides independent sequence data from millions of individual DNA molecules (**Figure 2**), allowing each fragment to be classified independently.

This unique ability extends upon the advantages of current molecular methods by allowing us to catalog the organisms present within even very complex polymicrobial bacterial communities, directly from patient specimens.

Information on Available Assays

Our lab currently offers high-fidelity Illumina next-generation DNA sequencing of clinical specimens which contain multiple bacterial DNA templates. Methods are validated for the purpose of clinical molecular diagnosis and patient care. Special rates are available for 16S rRNA research sequencing services and/or metagenomic analysis services.

To learn more about clinical applications and methodology, please see the following publications from our lab:

- Co-infection of *Fusobacterium nucleatum* and *Actinomyces israelii* in mastoiditis diagnosed by next-generation DNA sequencing
- Molecular diagnosis of *Actinomadura madurae* infection by 16S rRNA deep sequencing

Contact molmicdx@u.washington.edu for details or questions.

Clinical Reporting

Upon completion of testing, a report is issued describing the results of 16S next generation sequencing. To view a sample report, click here or the thumbnail at left.

For additional information on how to submit a request and recieve a report, please contact us!

Home / Available Tests / 16S Next Generation Sequencing

ILLUM-3871

Exhibit 408

Stanford HEALTH CARE
STANFORD MEDICINE

PATHOLOGY & LABORATORY MEDICINE

Anatomic Pathology & Clinical Laboratory Departments

Anatomic Pathology & Clinical Laboratory Departments

- Anatomic Pathology
- Biochemical Genetics
- Chemistry/Special Chemistry
- Coagulation
- Cytogenetics/FISH
- Flow Cytometry
- Hematology
- Microbiology
- Molecular Genetic Pathology
- Pediatric Pathology
- Point of Care
- Phlebotomy Services
- PreAnalytical (SHC and Hillview)
- RBC Special Studies
- Send Outs
- Special Coagulation
- Transfusion
- Virology

Exhibit 409

UNIVERSITY OF WASHINGTON DEPARTMENT OF LABORATORY MEDICINE
Molecular Diagnosis Microbiology Section

Menu

Available Tests
Submitting a Specimen
Order Form (PDF)
Acceptable Specimens
Clinical Significance
Contact Us
Publications/Links
About Us
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The molecular microbiology division offers a wide range of testing for identification of specific microorganisms and gene products, as well as detection of microbial DNA directly from clinical samples. Click on a test type in the box below for additional information.

More information regarding submission of samples to our lab for testing can be obtained viewing the Laboratory Medicine Online Test Guide or by clicking on the test codes (in gold). For additional details, please refer to our instructions on "How To Submit a Specimen."

Identification of cultured organisms by DNA sequence based methods: DNA of the cultured organism is isolated and amplified using universal primers and conventional PCR conditions. Amplified products are then sequenced and the organism is identified on the basis of the sequence data. Select a test type below to learn more.

- Bacterial Sequencing BCTSEQ
- Acid-Fast Bacilli (AFB) Sequencing AFBSEQ
- Yeast Sequencing YSTSEQ
- Mould Sequencing MLDSEQ

Direct detection of microbial DNA from tissues: DNA is isolated from specimen and amplified by conventional PCR using a battery of universal primers dependent on the test type requested. Amplified products are sequenced and the organism(s) identified on the basis of sequence data. Select a test type below to learn more.

- Detection of Bacterial DNA BCTPCR
- Reflexive Next-Generation Bacterial DNA Detection NGS16S
- Detection of AFB DNA NTMPCR and TBCPCR
- Detection of Fungal DNA FUNPCR

Detection and identification of specific pathogens: DNA is isolated from a cultured organism or direct patient specimen and amplified using primers developed specifically to detect a particular organism. Amplifications are performed either as conventional or real-time PCR. Select a test type below to learn more.

- Aspergillus PCR - BAL ASPPCR
- Aspergillus PCR - Tissue ASPTIS
- Cryptococcus PCR CRYPCR
- Coccidioides PCR COCPCR
- Histoplasma PCR HISPCR
- Pneumocystis PCR PNEPCR
- Zygomycete PCR ZYGPCR
- Toxoplasma PCR TOXPCR
- Bartonella PCR - Tissue or Culture BRTPCR
- Mycobacterium tuberculosis complex PCR - Tissue TBCPCR
- Nontuberculous Mycobacteria (AFB other than MTB Complex) PCR - Tissue NTMPCR
- Tropheryma whipplei PCR - Tissue TWHPCR
- Mycoplasma and Ureaplasma MPNPCR GUMPCR MSMPCR

Specific gene detection in cultured organisms: DNA of a cultured organism is isolated and amplified using primers developed specifically to detect a particular gene. Amplifications are performed under real-time PCR conditions and results are based on the presence or absence of amplification. Select a test type below to learn more.

- Staphylococcus aureus specific gene (sa442) SAPCR
- mecA gene MECPCR
- Rapid Mycoplasma Identification MPLS

Specific gene detection in direct specimens: DNA is isolated from the specimen and amplified using primers developed specifically to detect a particular gene. Amplifications are performed under real-time PCR conditions. Select a test type below to learn more.

- Clostridium difficile Toxin B gene from Stool CDTP

Organism typing: Select a test type below to learn more.

- MRSA Typing MRSATP

Home / Available Tests

ILLUM-3873

Exhibit 410

UCSF Developing NGS-based Infectious Disease Dx; Tests Metagenomic Sequencing on MinIon

Feb 20, 2015 | Monica Heger

Premium

SAN FRANCISCO (GenomeWeb) – Acute infectious diseases often go undiagnosed, including nearly half of patients with a diarrheal disease and as many as 60 percent to 80 percent of patients with encephalitis.

But, researchers at the University of California, San Francisco hope to improve the rate of infectious disease diagnosis —which would theoretically lead to better treatments and outcomes — using a metagenomic shotgun sequencing approach. This week at Cambridge Healthtech Institute's Molecular Medicine Tri-Conference, Charles Chiu, director of UCSF's Viral Diagnostics and Discovery Center presented details of an assay that he said would be launched initially as a laboratory-developed test in the next three to six months with an eye toward US Food and Drug Administration clearance.

In addition, he said that the laboratory has been exploring the use of nanopore sequencing using Oxford Nanopore Technologies' MinIon.

The metagenomic test is currently being developed on Illumina's MiSeq system and the lab is testing a variety of library prep approaches, including Illumina's Nextera XT and Rubicon Genomics' ThruPlex. It has developed an in-house bioinformatics pipeline, dubbed SURPI for sequence-based ultra-rapid pathogen identification, which helped decrease analysis time to between 10 minutes and 15 hours from several days. Analysis time is highly dependent on the complexity of the sample type and the analysis mode itself—there is a faster mode for viral and bacterial identification only or a more comprehensive mode that will align to all sequences in GenBank, Chiu explained.

The lab has diagnosed unsuspected parasites and viruses that other techniques failed to uncover, including neuroleptospirosis in a critically ill 14-year-old boy, a case that was published last year in *The New England Journal of Medicine*.

The test is "an all encompassing method to capture the full spectrum of agents," Chiu said during his presentation. The team uses a metagenomic shotgun sequencing approach of all the DNA or RNA present in a clinical sample. That unbiased approach enables the team to detect and reconstruct the sequences of "nearly any and all pathogens, including viruses, bacteria, fungi, and parasites," Chiu said.

Chiu's team has so far tested it on 50 patients with an infectious agent of unknown origin who had already received extensive testing to no avail. Of those, the team's metagenomic sequencing test was able to find a causative infectious agent in about 20 percent, Chiu said. Although "our yield was not as high as we'd like it to be," Chiu said, at a 30-day follow-up of the patients that did not receive a diagnosis, none had been found to have an infectious agent, suggesting that the assay did not miss anything.

Some of those patients were later diagnosed with an autoimmune disease, a form of vasculitis, or some other non-infectious cause, while some remained undiagnosed, Chiu said.

That initial patient population was "extremely biased," Chiu said, as they had already received a battery of tests, and in some cases had been put on antibiotics or other therapies.

In one case, a 75-year-old female complained of a fever that had lasted two months, abdominal pain, nausea, and vomiting. She previously had a kidney transplant and was found to have necrotic lymph nodes as well as the fungi *Candida albicans*. Next-gen sequencing diagnosed histoplasmosis.

Another case was a 20-year-old woman with a fever who had traveled to Australia, where there had been a recent outbreak of Ross River Virus. The patient had fever, headache, and muscle and joint pain. Lab tests were "unremarkable," including negative results for Ebola, HIV, and cytomegalovirus. However, there is no test in the US for Ross River Virus. Sequencing uncovered a "clear signature" of human herpesvirus 7, which Chiu said was unusual because most adults are immune to it. However, the antibody test was negative, suggesting it was a primary infection. They were able to rule out an unusual tropical virus and she recovered on her own in around 2 weeks.

A third case was also someone who had recently traveled. A 70-year-old male had fever, enlarged liver and spleen, and liver cysts and had tested negative in an infectious disease workup. He had recently gone on a six-week hiking trip in Spain and "remarked that he was bitten hundreds of times by insects." The NGS test uncovered a visceral leishmaniasis, which is a parasite acquired from sand fly bites. He was treated intravenously with an anti-fungal for two weeks and improved.

Chiu said that the lab is continuing to work on validating the test and locking down the protocol, after which it will begin offering it as an LDT. He said that the cost has not been set, but will likely be in the $300 range.

In addition, Chiu said the lab is working on a second version of SURPI, SURPIviz, which will speed turnaround time even more and include visualization features such as a heat map of the results.

Enhancements will also include rapid filtering of misannotated sequences, tagging of metadata to track background contamination, front-end visualization tools and a web-based interface, and taxonomic classification against all the sequences in GenBank. Under the current version of SURPI, analyzing 10 million reads takes around 40 minutes, but with the enhancements that will be reduced to five minutes, Chiu said.

In the future Chiu hopes to bring the test through FDA clearance. That test would run on the MiSeqDx. He said he is in discussions with the FDA about what would be required, and said it could include bringing the bioinformatics and analysis software through clearance.

Chiu anticipates that the LDT would run out of a core reference laboratory, at least initially, as

opposed to individual hospitals. Aside from applications in diagnostics, he said it may also have utility in public health surveillance, like outbreak tracking, and he said he is currently working with the US Centers for Disease Control and Prevention.

In the research setting, Chiu's lab has been testing metagenomic sequencing on Oxford Nanopore's MinIon. He declined to provide details about results, which he said would be published soon in a peer-reviewed journal, but said that he thinks "there is a lot of potential for real-time metagenomics sequencing [on the MinIon], and we're exploring potential applications of this to do point-of-care sequencing."

Filed Under Infectious Disease | Informatics | Clinical Sequencing | Molecular Diagnostics | NGS

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Next-generation Sequencing Pipeline Finds Cause of Critically Ill Boy's Disease

Jul 03, 2014
UCSF-led Team Develop Rapid Computational Pathogen Identification Pipeline for Clinical Use

Dec 08, 2014
Researchers Use Oxford MinIon, Illumina Sequence Data to Characterize Antibiotic Resistance Island

Sep 16, 2014
Oxford Nanopore Presents Details on New High-throughput Sequencer, Improvements to MinIon

Sep 09, 2014
MinIon Review by Early Access User Suggests Technology Not Ready for Routine Use Yet

Jan 14, 2015

CSHL Scientists Sequence Yeast Genome with Oxford Nanopore MinIon

Exhibit 411

An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1, HBV, and HCV

Dmitry A. Khodakov[1], Natalia V. Zakharova[1], Dmitry A. Gryadunov[1], Felix P. Filatov[2], Alexander S. Zasedatelev[1], and Vladimir M. Mikhailovich[1]

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We describe a novel microarray-based approach for simultaneous identification and quantification of human immunodeficiency virus type 1 (HIV-1) and hepatitis B and C viruses (HBV and HCV) in donor plasma specimens. The method is based on multiplex real-time RT-PCR performed within the microarray hydrogel pads. Double-stranded amplification products are simultaneously detected using nonspecific SYBR Green I dye due to the reaction run in separate pads bearing 5′-immobilized specific primers. Both the sensitivity and specificity of the assay, based on 132 blood specimens analyzed, were 100% (56, 26, and 8 specimens were seropositive to HBV, HCV, and HIV-1, respectively; 22 were positive to both HIV-1 and HCV, and 2 positive to all three viruses; 18 samples were pathogen-negative). The dynamic range of the quantitative analysis covered a six-order interval ranging from 10^0 to 10^6 genome equivalents per assay. The 95% detection limits were 14 gEq for HIV-1, 10 gEq (1.7 IU) for HBV, and 15 gEq (7.5 IU) for HCV per assay. The proposed approach is considered to be versatile and could be adapted for simultaneous identification and quantification of numerous genetic targets.

INTRODUCTION

The risk of transfusion-transmitted infection with hepatitis B and C viruses (HBV and HCV, respectively), and human immunodeficiency virus type 1 (HIV-1) can be significantly decreased if fast, sensitive, and reliable methods are used to identify these pathogenic viruses in donor blood. Although essential progress has been made in the development of immunological methods (1,2), their application remains limited during the seronegative window period as well as in cases of delitescence and immunovariant and nonimmunogenic forms of infections (3), which are inherent, in particular, in HBV, HCV, and HIV-1.

Methods based on nucleic acid testing (NAT) seem to be preferable for diagnostic applications since they are more sensitive and allow direct identification of specific fragments of infectious agent genome (4,5). Besides, the NAT methods could be performed in multiplex format to identify several agents simultaneously. At present, a number of commercial test systems are available for simultaneous detection of HIV-1, HBV, and HCV in donor blood (5–10). These systems are based on various technical approaches using several methods for sequence-specific amplification of viral nucleic acids, such as PCR (or RT-PCR), ligase chain reaction, nucleic acid sequence-based amplification (NASBA), and transcription-mediated amplification (TMA) (4).

The most advanced techniques are based on real-time detection, which essentially shortens the duration of analysis, improves its sensitivity, and at the same time allows quantitative determinations. Such systems do not require any post-amplification detection of the reaction products (electrophoresis and hybridization) and, thereby, substantially reduce the risk of contamination. There are several commercial systems developed for real-time NAT-analysis (11).

Most of these real-time systems detect amplification products using either nonspecific DNA binding fluorophores or specific fluorescently labeled oligonucleotide probes (5′-endonuclease, adjacent linear and hairpin oligoprobes, and self-fluorescing products) (11,12). Unfortunately, the real-time PCR used in multiplex reactions has an essential limitation: the interference of absorption and fluorescence spectra of simultaneously used fluorophores allows reliable detection of no more than four reactions in one assay (7). The use of nonspecific DNA binding fluorophores, such as SYBR Green I, is also limited, allowing for real-time detection of the total amount of all amplification products synthesized in in-tube reactions (13). This type of dye is commonly used for melting curves measurements providing qualitative analysis (14).

In an effort to further improve the real-time multiplex reaction systems, several variants of oligonucleotide immobilization on solid phase have been proposed, in particular, probe-coated microspheres (6, 15) and DNA microarrays (16–21).

[1]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences and* [2]*Hematological Research Center, Russian Academy of Medical Sciences, Moscow, Russia*

Oligonucleotide microarrays are powerful tools for parallel, high-throughput detection of numerous nucleic acid targets. Originally developed for the analysis of whole genome gene expression and micro-sequencing, DNA microarrays have considerable potential for microbial and viral diagnostics (22–24). A number of various approaches from routine hybridization to sophisticated enzymatic assays have been developed on microarray platforms (25). One of the most promising approaches to pathogen detection and identification seems to be real-time multiplex PCR performed on a microarray.

DNA microarray with immobilized probes can increase the number of simultaneously analyzed targets (26). At the same time, all microarrays with immobilized primers developed so far use post-amplification detection of results only (19–21).

In this study, an original on-chip real-time PCR approach for simultaneous identification of several genetic targets is described. We have developed a procedure of the on-chip quantitative amplification and applied it to the identification of blood-borne viruses in plasma specimens. This approach is fundamentally different from other multiplex systems since it provides real-time PCR with primers immobilized on a microarray and use a single detector dye. The developed approach combines high analytical sensitivity with multiplex format and can be considered as a versatile tool for further development of other test systems for simultaneous identification of various genetic targets.

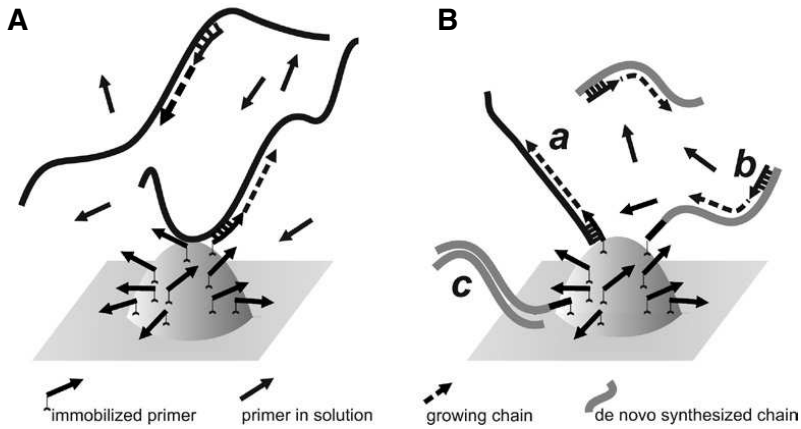

Figure 1. Scheme of real-time PCR inside amplification volume of a gel-based microarray. Forward primers are immobilized, while reverse primers are free in surrounded solution. (A) At the initial reaction stage when the concentration of the target molecules (shown by black bold lines) is low, specific amplification products are formed through the extension of both free and immobilized primers. (B) At the exponential stage, the extended immobilized primers (*a*) form the additional anchored templates to be amplified with free reverse primers (*b*). Double-stranded de novo synthesized product shown by gray lines (*c*) binds SYBR Green I. The accumulation of the dye in the gel pad is detected during each cycle of amplification.

MATERIALS AND METHODS

Primer Design and Microarray Fabrication

The HIV-1 *gag* gene, HBV *X*-gene, and HCV 5′-untranslated region (5′-UTR) sequences available from public databases were analyzed with BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) software. Based on multiple sequence alignments, PCR primers (Table 1) were derived from these regions using the Oligo 6 software (Molecular Biology Insights, Cascade, CO, USA). The sequence identities between the designed primers and the genes of interests were analyzed with a BLAST search (www.ncbi.nlm.nih.gov).

Primers were synthesized on an ABI-394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) using standard automated phosphoramidite chemistry. To immobilize primers inside hydrogel pads, an amino group was introduced during synthesis using 5′-Amino-Modifier C6 (Glen Research, Sterling, VA, USA). Primers were purified by reverse phase HPLC

Table 1. Specific Primers Used in Two-stage PCR of HIV-1, HBV, and HCV

Primer[a]	Positions[b]	Sequence 5′→3′
HIV_OUT_F	609	AGAACCG(G/A)TCTACATA(G/A)TCTCTAA(G/A)G
HIV_OUT_R	872	TGAGGA(G/A)GCTGCAGAATGGGA
HIV_IN_F_I	643	TGG(C/T)CCTTGT(C/T)TTATGTCCA(G/A)AATG
HIV_IN_R	801	AGAACCAAGGGGAAGTGACATAG
HBV_OUT_F	1373	TCCATGGCTGCTAGGCTGTG
HBV_OUT_R	1584	GTGCAGAGGTGAAGCGAAGTG
HBV_IN_F_I	1431	GTCCCGTCGGCGCTGAATC
HBV_IN_R	1528	CGCGTAAAGAGAGGTGCGCC
HCV_OUT_F	69	CAGAAAGCGTCTAGCCATGGCGT
HCV_OUT_R	290	ACTCGCAAGCACCCTATCAGGCA
HCV_IN_F_I	146	GTCTGCGGAACCGGTGAGTACA
HCV_IN_R	268	GTACCACAAGGCCTTTCGCGAC

[a] OUT, first-stage PCR primers (outers); IN, second-stage on-chip PCR primers (inners); F, forward; R, reverse; I, immobilized.
[b] The number indicates nucleotide position, counting from the 5′ terminus of the gene-encoding sequence according to Gen Bank accession no. AY206658 (HIV-1, *gag* gene, cDNA), AB126581 (HBV, *X*-gene), and AF009606 (HCV, 5′-UTR region).

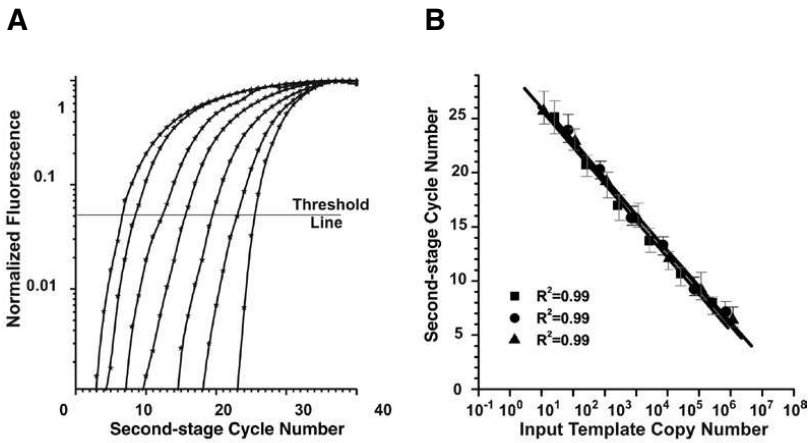

Figure 2. Second stage of on-chip real-time amplification of standard plasma samples. (A) Plot of fluorescence accumulation and threshold cycle number (C_T) for HBV DNA serial 10-fold dilutions (log10) ranging from 10^6 to 10^0 gEq/mL (from left to right). Threshold fluorescence is shown as the horizontal line. (B) Calibration curves to calculate initial nucleic acid concentrations of HIV-1, HBV, and HCV in plasma samples (curves marked with squares, triangles, and circles, respectively). Regression coefficient for each target is indicated.

on C18-Nucleosil (5 µm, 4.6 × 250 mm) columns (Sigma-Aldrich, St. Louis, MO, USA).

Microarrays for real-time PCR were manufactured as described earlier (27). High porosity hydrogel M18 (Biochip-IMB Ltd, Moscow, Russia) was used to form microarray hydrogel pads 600 µm in diameter. The primers were immobilized in the pads at their 5′ ends at 200 µM.

Reference Specimens and Clinical Samples

Reference serology positive plasma samples, in which HIV-1, HCV, and HBV viral loads had been quantified, were kindly provided by Jacques Izopet (Institut Fédératif de Biologie—Hôpital Purpan, Toulouse, France). The quantitative plasma standards contained 3.85 × 10^6 gEq/mL of HIV-1, 1.73 × 10^7 gEq/mL (2.89 × 10^6 IU/mL) of HBV, and 1.04 × 10^7 gEq/mL (5.22 × 10^6 IU/mL) of HCV.

The plasma standards and their serial dilutions prepared using seronegative plasma were used to generate calibration curves and evaluate the 95% detection limits. To produce calibration curves, 10-fold dilutions were performed to cover the range from 10^6 to 10^0 gEq per assay. For concentrations of 10^2 gEq/10 µL and higher 4 to 8 replicates were used; lower concentrations were tested in 25 replicates.

To calculate the 95% detection limits, serial dilutions were prepared in concentrations ranging from about 10^2 to 1 gEq/10 µL by means of 2-fold dilutions. Each point was tested in 25 replicates.

Plasmids pBluescript II (Stratagene, La Jolla, CA, USA) with cloned fragments HG00-HG01 of the HIV-1 *gag* gene (strains VI310 and TB132) were used as control templates to explore the PCR assay characteristics.

Anonymous plasma samples were collected at the Moscow Hematological Research Center and serologically characterized. In total, 132 randomly selected samples were tested: 18 seronegative, 56 HBV-positive, 26 HCV-positive, 8 HIV-1–positive; 22 samples were positive to both HIV-1 and HCV and 2 were positive to HIV-1, HBV and HCV.

Viral DNA/RNA Isolation

Viral nucleic acids were isolated from 200 µL of plasma samples with QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Nucleic acids were eluted with 30 µL of AVE buffer, and then were either directly analyzed or stored at -70°C until further use.

Multiplex RT-PCR

A reaction mixture for the first stage of multiplex RT-PCR contained 1× StrataScript buffer (Stratagene); 0.2 mM each dATP, dCTP, and dGTP; 0.6 mM dUTP; 0.2 µM of each first-stage primer (Table 1); 5 U SureStart Taq polymerase (Stratagene); 10 U StrataScript Reverse Transcriptase (Stratagene); 20 U RNasin (Fermentas, Vilnius, Lithuania); and 10 µL of a template nucleic acid preparation per 25 µL reaction. The RT-PCR was performed as follows: the RT reaction at 55°C for 30 min, Taq polymerase activation at 95°C for 10 min, 25 three-step cycles (20 s at 95°C; 30 s at 62°C; and 30 s at 72°C), and final incubation at 72°C for 5 min.

Real-time PCR on Microarray

The second-stage real-time PCR was performed on the microarray with the corresponding forward immobilized primers (Table 1, Figure 1) as described earlier (28) with some modifications. In brief, amplification was performed in the presence of Brilliant SYBR Green Quantitative PCR Core Reagent kit (Stratagene). A reaction mixture (25 µL) contained: the core PCR buffer; 4 mM $MgCl_2$; 0.2 mM each dATP, dCTP, and dGTP; 0.6 mM dUTP; 0.2 µM of each second-stage reverse primer (Table 1); 5 U SureStart Taq polymerase; 1× SYBR Green I; 0.01% acetylated BSA (New England BioLabs, Ipswich, MA, USA); and 1 µL of the first-stage product as a template. The reaction was carried out in a microchip chamber formed between the microchip slide, a cover glass, and a Frame-Seal (Bio-Rad Laboratories, Hercules, CA, USA) between them. After an initial incubation at 95°C for 10 min, 30–50 three-step cycles were performed as follows: 30 s at 95°C, 30 s at 55°C, and 45 s at 68°C.

A homemade experimental setup was used for fluorescence measurements. The setup consisted of fluorescence microscope assembled with thermocycler, a charge-coupled device (CCD), camera, and the specialized

software ImageExpress (Biochip-IMB). Fluorescence signals in gel pads were measured in real-time format at 3 s before the end of each elongation step.

The post-PCR melting curves were obtained by increasing temperature from 68° to 95°C at the rate of 3°C/min. To obtain the melting curves, the fluorescence was measured at 0.5°C interval each 10 s during the melting process.

The rough fluorescence data (F) were analyzed by ImageExpress software and normalized according to the formula $(F\text{-}F_{min})/(F_{max}\text{-}F_{min})$, where F_{min} and F_{max} are minimal and maximal fluorescence values registered in a gel pad.

On-chip Quantification of Viral DNA/RNA in Clinical Samples

To quantify HBV DNA, and HCV and HIV-1 RNA in clinical plasma samples using on-chip real-time PCR, the C_T (threshold cycle) value determined for each sample was interpolated to the calibration curves generated as described above. At least three replicates of each specimen were tested.

RESULTS

Design of the Assay

An original approach based on real-time PCR on oligonucleotide microarray of isolated gel drops of nanoliter volume was developed to identify and simultaneously quantify HIV-1, HBV, and HCV in blood samples. The microarray consisted of hemispheric hydrogel pads with 5′-immobilized forward primers. Primer sequences are listed in Table 1. The HIV-1 *gag* gene, HBV *X*-gene, and HCV 5′-UTR were selected as specific targets to be identified.

The developed technique included two stages: (*i*) pre-on-chip multiplex RT-PCR (see Materials and Methods) and (*ii*) multiplex on-chip PCR combined with real-time fluorescence measurements and data acquisition. The first RT-PCR stage performed in a tube was aimed at obtaining cDNA, increasing the reaction sensitivity and specificity due to the nested format, and optimizing the dynamic range of quantitative analysis that could be shifted by variation of RT-PCR stage cycle number (see Quantitative On-chip Real-time PCR section, below).

The use of specific primers immobilized in hydrogel pads to perform on-chip PCR was described earlier (27). In this study, another approach is proposed. It supposes no specific fluorescent labels are incorporated into the in-target molecules. Identification of double-stranded amplification products in hydrogel pads is carried out via fluorescence detection of nonspecific dye SYBR Green I that binds and intercalates into the products. The scheme of the reaction is illustrated in Figure 1.

Products created at the RT-PCR stage are used as templates for real-time on-chip PCR. Target DNA molecules anneal to specific primers immobilized in individual gel pads. Figure 1B shows how the immobilized primers are extended enzymatically (*a*), forming new target chains anchored in the gel via their 5′- termini. These chains serve as targets for further amplification (*b*), and eventually form tethered double-stranded hybridization complexes (*c*) that could bind fluorescent dyes. Since each gel pad contains primers specific to one target only, the kinetics of fluorescence in each gel pad reflects the accumulation of the corresponding amplification product.

Fluorescence signals in gel pads were measured at the end of each elongation step, when specific double-stranded hybridization complexes remain stable due to their high melting temperature, in contrast to shorter nonspecific products. As a result, only specific double-stranded products bind the SYBR Green I dye. To make sure that amplification products produced by on-chip PCR are specific, additional melting curve analysis inside individual gel pads was carried out. The emergence of complexes with high melting temperature within the gel pad indicates that the reaction of amplification was successful and specific.

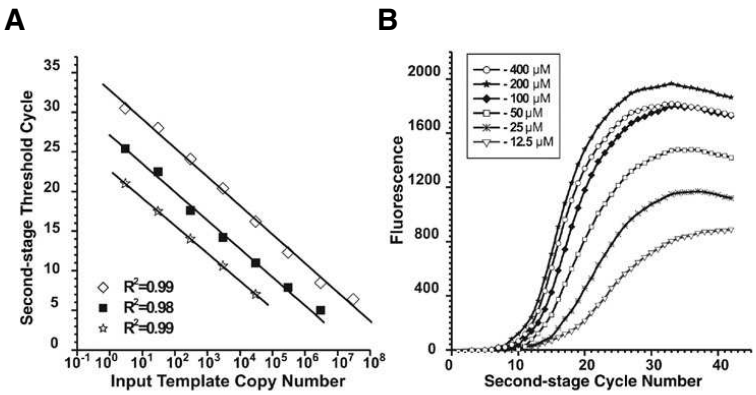

Figure 3. On-chip real-time PCR. (A) Effect of the cycle number at the first RT-PCR stage on calibration curves characteristics. Plot of C_T vs. serial dilutions obtained after 20 (open stars), 25 (closed squares), and 30 (open diamonds) cycles of the first-stage PCR. The *gag* gene of HIV-1 (strain TB132) was cloned in Bluescript II (Stratagene, La Jolla, CA, USA) vector and 10-fold serial dilutions ranging from 3 to 3×10^7 gEq per assay were used as targets. (B) Influence of immobilized primer concentration on the on-chip amplification efficiency. Amplification curves were obtained at indicated concentrations of immobilized primer. The *gag* gene of HIV-1 (strain VI310) cloned in Bluescript II vector (10^5 gEq per assay) was used as a target.

Quantitative On-chip Real-time PCR

To produce calibration curves, serial 10-fold dilutions of the standard plasma samples were tested (see Materials and Methods). The correlation between the threshold cycle number (C_T) and template copy number is shown in Figure 2. The C_T values determined from the plots similar to those shown in Figure 2A were used to obtain the calibration curves presented in Figure

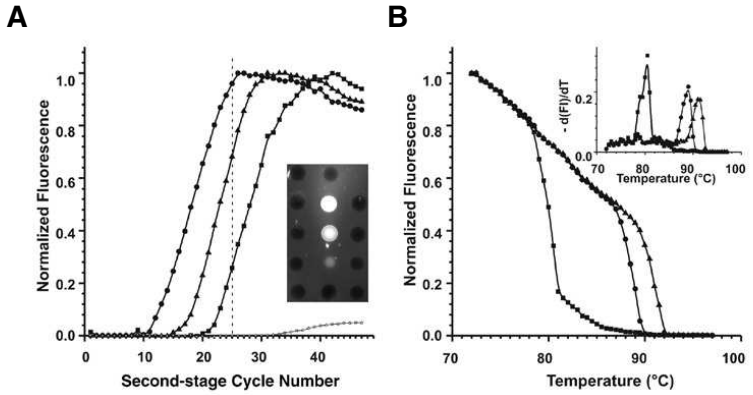

Figure 4. Second-stage real-time on-chip amplification of HCV, HBV, and HIV-1 targets and melting of the amplification products. (A) Amplification plot produced with ImageExpress software (Biochip-IMB Ltd, Moscow, Russia) and fluorescence image of the microarray. The plasma sample contained a mixture of 9×10^3 gEq/mL of HCV (circles), 10^2 gEq/mL of HBV, and 10^1 gEq/mL of HIV-1 (triangles and squares, respectively). The insert shows fluorescence accumulation in gel pads corresponding to HIV-1 (top bright gel element), HBV (middle element), and HCV (bottom bright element) after 25th cycle. Gel pads with immobilized primers are surrounded by background control gel pads. The increase of fluorescence in control pads is marked by the curve with open stars. (B) Melting of duplexes formed between immobilized extended primers and single-stranded amplification products. Melting peaks are shown on the corresponding differentiation plot in the insert.

2B. The 95% detection limits were 14 gEq for HIV-1, 10 gEq (1.7 IU) for HBV, and 15 gEq (7.5 IU) for HCV per assay. The results were reproducible: the deviations in the logarithmic concentration values were within 1Log when four to eight replicates were tested for each concentration.

The dynamic range of quantitative analysis covered six orders of concentrations from 10^0 to 10^6 gEq of HIV-1, HBV, and HCV per reaction if the number of cycles of the first RT-PCR stage was 23–28. Decreasing the number of preliminary stage cycles, one could shift the dynamic range toward quantifying higher concentrations. If the number of pre-on-chip amplification cycles was more than that mentioned above, the dynamic range got narrower but the sensitivity did not increase (see Figure 3A).

The concentration of immobilized primer was shown to be essential for both on-chip PCR efficacy and absolute fluorescence signal intensity. Figure 3B demonstrates the results obtained for various primer concentrations ranging from 12.5 to 400 µM. No significant difference in amplification efficacies was found when 100, 200, or 400 µM concentrations were used. Meanwhile definite decrease of absolute fluorescence signals and increase of C_T value were observed at concentrations below 100 µM. Thus, the concentration of 200 µM was chosen for further assays.

The SYBR Green I concentration suggested by the manufacturer (1×) proved to be optimal to monitor on-chip PCR. Although a 3-fold increase in the dye concentration led to some growth of absolute fluorescence, the efficacy of PCR decreased significantly due to inhibition of the enzyme activity (data not shown).

As a result of optimization, we defined conditions for highly effective on-chip PCR. Overall, its sensitivity did not concede that performed in tubes.

Simultaneous On-chip Real-time Amplification of HIV-1, HBV, and HCV

Figure 4A represents simultaneous on-chip real-time PCR of HIV, HBV, and HCV targets. To demonstrate various kinetics of product accumulation, the three targets were tested in different concentrations. The microarray image was taken at the end of 25th elongation cycle. The T_m values calculated by the melting curve analysis (Figure 4B) were 80°, 92°, and 89°C for HIV-1, HBV, and HCV amplification products, respectively. The obtained temperatures coincided with those calculated theoretically.

To evaluate the ability of the multiplex assay to determine the concentration of each target in the presence of excessive amounts of other viral nucleic acids, serial dilutions of each target (ranging from 10^1 to 10^5 gEq) were analyzed in the presence of about 10^5 gEq of two other viruses. The assays were performed using all possible combinations of the three viruses. No interference between the unrelated viruses and the calibration curve obtained for the tested virus was observed in any combination (Figure 2B). The calibration and melting curves remained unchanged regardless of whether one, two, or three targets were tested in the reaction simultaneously.

Testing Clinical Plasma Samples by Real-time On-chip PCR

A total of 132 serologically characterized blood samples (see Materials and Methods) were analyzed by the developed method. The viral load values indicated below correspond to quantities per 1 mL of initial clinical specimens.

The estimated range of viral loads in 32 HIV-1 serologically positive samples was from 60 to 4.2×10^7 gEq/mL. Five of them contained <1000 gEq/mL.

Among 58 HBV positive samples, 11 had viral loads <1000 gEq/mL and 7 samples had loads $>1.0 \times 10^7$ gEq/mL. The minimal determined load was 50 gEq/mL (8.3 IU/mL).

The minimal load identified in HCV-containing specimens ($n = 50$) was 550 gEq/mL (275 IU/mL); 9 samples had values <1000 gEq/mL; and 4 had loads $>1.0 \times 10^7$ gEq/mL.

In 4 out of 22 samples containing both hepatitis viruses and in 1 out of 2 specimens containing all 3 viruses (HIV, HBV, and HCV), viral loads within 1 sample varied in a range of up to 4 orders of magnitude. With at least 3 replicates of each sample isolated and analyzed separately, the deviations for identical sample portions remained within 1Log of concentrations, thus confirming the reproducibility of the quantitative results.

Eighteen Seronegative Samples Were Tested as Negative Controls, and No False-positive Results Were Obtained

Twenty randomly selected samples were encrypted and tested in a blind format. All positive specimens were correctly identified by on-chip PCR, while no specific amplifications were observed for seronegative samples.

The results of qualitative analysis obtained using serological methods and on-chip PCR techniques were in full concordance. In this study, we had no aim to compare quantitative results for clinical specimens.

DISCUSSION

The paper describes an original approach for simultaneous quantitative identification of HIV-1, HBV, and HCV in blood plasma specimens. The method uses a gel-based oligonucleotide microarray platform to run multiplex PCR in real time. The use of three-dimensional (3-D) polyacrylamide provides a number of advantages (29,30); in particular, it may enhance DNA polymerase activity with immobilized primers relative to primers immobilized on a two-dimensional (2-D) surface (20).

Due to complete physical isolation of individual gel elements, it is possible to use a single nonspecific DNA binding dye (SYBR Green I) to detect all of the multiplex reaction products simultaneously and independently using a microarray consisting of a set of primers immobilized inside separate gel pads. Spatial separation of the immobilized specific primers on a microarray allows the real-time identification of specific targets by measuring the fluorescence emanated from individual gel pads in the course of PCR. This feature distinguishes the proposed approach from other multiplex systems that use SYBR Green I for indirect melting curves analysis of the total amplification product (14). Besides, this technique does not require the preparation of additional specific probes, such as TaqMan (26) or dyes varied in their emission spectra (12).

The technique was developed for the detection of HIV-1, HBV, and HCV because of the necessity of their rapid and reliable identification in blood plasma for early recognition of the socially and epidemiologically dangerous diseases and for large-scale analysis of blood donations. According to the evaluations developed in the UK, USA, and Japan, the use of the NAT-methods for testing donor blood has decreased the risk of HBV, HCV, and HIV-1 transmitting by 90%, 95%, and 80%, respectively (3,31,32). Although several commercial systems are available for simultaneous NAT-testing of HIV-1, HBV, and HCV (5–10), further development of responsive, specific, and reliable multiplex techniques remains an important goal for their wide routine application.

In this study, the design of primers set was carried out to take into account every known polymorphism in the genomes of the viral subtypes. To succeed in amplification of the majority of virus subtypes, the standard primer degeneration tactics was applied. It is admitted that some modifications in primer sequences could be required after testing more various genotypes. Nevertheless, these minor modifications should not affect the general concept of this work.

A nested PCR strategy was applied to increase the sensitivity and specificity of the developed approach. In the course of our experiments, we found that by varying the first-stage PCR cycle number the dynamic range of the method could be easily shifted to desirable range without other key modifications of the procedure. Excellent accordance of the calibration curves obtained for HIV-1, HBV, and HCV when the quantities of the targets are expressed in genome equivalents strongly suggests equal efficacy of amplification of all three targets.

During the optimization of the described assays, we encountered consistent and significant shifts of the optimal annealing temperatures relative to theoretical calculations, when PCR is performed within the microarray gel pads. Adjustment of the theoretically calculated annealing temperature of the immobilized primers has resulted in increased efficiency of real-time PCR. As we described earlier, the annealing temperatures of immobilized primers are approximately 5°C lower than those calculated for primers in solution (28). Although the chemistry of the gel used for the manufacturing of microarrays in this work is different from our previous publications, and the sequences of the primers are completely new, the temperature shift remains similar. As a result, the yield of on-chip PCR products was higher if the annealing temperature was decreased by 5°–6°C in comparison with that calculated theoretically.

The data obtained in this study concerning the identification and quantification of viruses in standard specimens, as well as in clinical plasma samples, show that the developed biochip-based approach is comparable in sensitivity to existing systems built on other principles and used for testing donor blood for HIV-1, HBV, and HCV (5–10). In contrast to a recently developed microarray (24), our approach allows simultaneous identification and quantification of three different viral targets. The assay can be applied for the analysis of samples from patients who have high co-infection rates of blood-borne viruses.

We conclude that the described method is sensitive, specific, and relatively easy to use. The biochip-based analysis can serve as a versatile basis for future development of diagnostic systems for large-scale analysis of clinical blood samples. The developed conception can also be applied for accomplishment of a wide spectrum of tasks related to the multiplex identification and quantification of other genetic targets.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Dmitry A. Khodakov, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov St., 119991 Moscow, V-334, Russia. e-mail: hodamid@biochip.ru

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Microarray multiplex assay for the simultaneous detection and discrimination of hepatitis B, hepatitis C, and human immunodeficiency type-1 viruses in human blood samples ☆

Chu Chieh Hsia [a,*], Vladimir E. Chizhikov [b], Amy X. Yang [c], Angamuthu Selvapandiyan [a], Indira Hewlett [a], Robert Duncan [a], Raj K. Puri [c], Hira L. Nakhasi [a], Gerardo G. Kaplan [a]

[a] *Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Review, Food and Drug Administration, Bethesda, MD 20892, USA*
[b] *Division of Viral Products, Center for Biologics Evaluation and Review, Food and Drug Administration, Bethesda, MD 20892, USA*
[c] *Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Review, Food and Drug Administration, Bethesda, MD 20892, USA*

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Abstract

Hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus type-1 (HIV-1) are transfusion-transmitted human pathogens that have a major impact on blood safety and public health worldwide. We developed a microarray multiplex assay for the simultaneous detection and discrimination of these three viruses. The microarray consists of 16 oligonucleotide probes, immobilized on a silylated glass slide. Amplicons from multiplex PCR were labeled with Cy-5 and hybridized to the microarray. The assay detected 1 International Unit (IU), 10 IU, 20 IU of HBV, HCV, and HIV-1, respectively, in a single multiplex reaction. The assay also detected and discriminated the presence of two or three of these viruses in a single sample. Our data represent a proof-of-concept for the possible use of highly sensitive multiplex microarray assay to screen and confirm the presence of these viruses in blood donors and patients.
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Hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus type-1 (HIV-1) are blood-borne viruses of worldwide public health concern.

The implementation of minipool nucleic acid amplification tests (NAT) for HCV and HIV-1 to screen blood and plasma donors reduced the risk of transmission of these viruses by blood and blood products [1,2]. The development of NAT for the simultaneous detection and discrimination of these three viruses (multiplex assays) could reduce the cost of blood screening and clinical diagnosis. Currently, there are two FDA-licensed duplex NAT assays to screen blood donors for HCV and HIV-1 [3]. Multiplex assay for simultaneous detection of these three viruses is under development [4,5]. But all these assays require further discrimination tests to determine the specific viral infection(s). Microarray technology has been used to study gene expression in clinical and biological samples, detect and genotype pathogens [6–11], detect single base pair mismatches [12], mapping genomic library clones [13], and study viral evolution [14]. The utility of this technology to simultaneously detect and discriminate multiple viruses has not been fully explored. Here, we describe the development of a highly sensitive and specific microarray-based multiplex assay that detects simultaneously HBV, HCV, and HIV-1 in human blood samples, without the need of

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* Corresponding author. Fax: +1 301 480 7928.
E-mail address: chuchieh.hsia@fda.hhs.gov (C.C. Hsia).

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additional discrimination tests. This test could be used to detect viruses in different body fluids or tissues from human or animals.

Materials and methods

Human plasma and sera samples. A total of 23 human plasma or sera samples were tested in this study. The following samples contained HBV: the WHO International Standard for HBV DNA NAT assay (NIBSC code No. 97/746 contains 1×10^6 IU/mL, South Mimms, UK), the CBER HBV DNA NAT panel (FDA, Bethesda, MD; genotype A), plasma from eleven HBV seroconversion panels (acute HBV infections, ZeptoMatrix Corp. Franklin, MA), and sera samples from 3 HBV chronic carriers and 3 HBsAg-negative HBV-DNA(+) hepatocellular carcinoma patients (Chinese Academy of Medical Sciences, Beijing, China). The following samples contained HCV: the WHO International Standard for HCV RNA NAT (NIBSC code No. 96/790; genotype 1a, contains 1×10^5 IU/mL), the HCV RNA Working Reagent for NAT (NIBSC code no. 98/576; genotype 3a, contains 7.1×10^2 IU/mL), and CBER HCV RNA NAT panel (FDA, Bethesda, MD; genotype 1b). Samples that contained HIV-1 were from the CBER HIV-1 RNA NAT panel (FDA, Bethesda, MD; group M, subtype B). Each member of these CBER RNA panels contains established number of genome equivalents (gEq)/mL RNA. Studies using human samples were approved by the FDA Research Involving Human Subject Committee (RIHSC) under protocol #06-0078.

Design of multiplex PCR primers and microarray probes. PCR primer pairs of 19–28 nucleotides in length and T_m of 52–60 °C that do not form dimers and hairpins were selected to amplify conserved regions in the S gene of HBV, the 5′ UTR of HCV, or the gp^{41} region of HIV-1. Primers were selected using Mac Vector 7.2 (Genetic Computer group, Madison, WI) and Primer 3′ software [15] (Table 1). The specificity of viral primers and lack of cross-hybridization with nucleic acid sequences of human or other origin were tested using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Our oligonucleotide microarray contained 20 spots (Fig. 1A). The 16 gene-specific probes contain 30–47 nucleotides and a T_m of 63–73 °C (Table 2).

RNA and DNA extraction, reverse transcription, and PCR. Nucleic acids from samples containing HBV, HCV, and/or HIV-1 were extracted using NucliSens reagents (BioMérieux, Boxtel, Netherlands) in the presence of 5 ng of human DNA/sample as an internal control. The extracted nucleic acids were reverse transcribed and amplified using SuperScript III One-step RT PCR Platinum *Taq* HiFi reagent mix (Invitrogen Corp. Carlsbad, CA), and the 1st PCR primers (Table 1). The 2nd PCR used AmpliTaq Gold enzyme, GeneAmp PCR buffer II, Mg Cl_2, GeneAmp dNTP mix, and the eight primers (Table 1). Both 1st and 2nd PCR were performed in a final volume of 50 μl for 40 cycles, on a 9600 or 9700 GeneAmp PCR System (PerkinElmer Life Sciences, Inc., Boston, MA).

Labeling of single stranded DNA (ssDNA). The 2nd PCR products (amplicons) were purified using the MinElute PCR purification Kit (Qiagen Inc. Valencia, CA, USA). They were labeled with indodicarbocyanine (Cy5)-dCTP (PerkinElmer Life Sciences Inc., Boston, MA) in a multiplex reaction containing Elmer the four reverse primers for 2nd PCR (Table 1). The Cy-3 antisense quality control oligonucleotide (QC), with sequences complementary to the QC (Table 2), was prepared by 5′-end labeling with indocarbocyanine (Cy3)-dCTP during synthesis. The Cy3-antisense QC was purified by high performance liquid chromatography (HPLC).

Microarray fabrication, hybridization, and scanning. Each gene-specific oligonucleotide probe was spiked with QC. Probes were printed on silylated glass slides (SSC aldehyde-slide, CEL Associates Inc., Pearland, Texas) using a contact microspotting robot (Cartesian Technologies, Inc.) and a ChipMaker microspotting device with a single CMP-7 pin (TeleChem International Inc.). Each slide was spotted with six sets of three arrays, then treated and stored as described previously [6]. The quality of each lot of printed slides was tested by hybridizing the last printed slide with Cy-3-labeled antisense-QC (Fig. 1A, right image). The Cy5-labeled amplicons from 2nd PCR and Cy3-labeled antisense QC were mixed with an equal volume of 2× hybridization buffer, hybridized on microarrays. The slides were incubated for 1.5 h at 45 °C in a water bath, finally were sequentially washed as reported (6). The slides were scanned on a GenePix 4000B Microarray Scanner (Molecular Devices Corp, Axon Instruments, Sunnyvale, CA), a laser-excitation-based epifluorescence scanning system. Scanning was performed at 635 nm to detect Cy5, and at 635/532 nm dual wavelength to detect Cy3 or a mixture of Cy5 and Cy3, respectively, using GenPro Pix 5.1 software (Molecular Devices Corp, Axon Instruments).

DNA sequencing and genotyping of HBV samples. A fragment of the S gene of HBV (241 bp) was amplified by nested PCR using primers S21–S24 [16] and sequenced in an automated sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems, Foster City, CA). The deduced amino acid sequences were aligned with the consensus sequences in

Table 1
Primers for microarray multiplex assay

Function and name		Target	Sequence (5′–3′)	Gene	Positions (nt)[a]	Amplicons (bp)
Viral specific primers						
1st PCR	IV-105 (F)	HIV	GGTTCTTGGGAGCAGCAGGAA		7786–7806	
	IV-106 (R)		GACAATGGTGAGTATCCCTGCCTAACT	gp^{41}	8345–8371	586
2nd PCR	IV-35 (F)		TCCTGGGGATTTGGGGTTG		7999–8017	
	IV-36 (R)		CTTGCTGGTTTTGCGATTC		8166–8184	186
1st PCR	CV-101 (F)	HCV	TCCCCTGTGAGGAACTACTGTCTTC		41–65	
	CV-102 (R)		TTGAGGTTTAGGATTCGTGCTC	5′-UTR	344–365	325
2nd PCR	CV-21 (F)		ATGGCGTTAGTATGAGTGTCGTRC		85–108	
	CV-22 (R)		CCCAAATCTCCAGGCATTGAG		211–231	147
1st PCR	S-61 (F)	HBV	ATCTTCCTGCTGGTGGCTC	Pre-S2	51–69	
	S-62 (R)		AAACGGGCAACATACCTTG		455–473	423
2nd PCR	BV-11 (F)		CAGTCCCCAACCTCCAATCAC	S	314–334	
	BV-12 (R)		GGCATAGCAGCAGGATGAAGAG		404–425	112
Internal control primers						
1st PCR	Hu-5 (F)	Human gene	TCGAAGACGATCAGATACCGT		1147–1167	
	Hu-4 (R)		TTGCAACCATACTCCCCCCG	18S rRNA	1264–1283	137
2nd PCR	Hu-5 (F)		Same as above			
	Hu-6 (R)		ATACTCCCCCCGGAACC		1259–1275	129

[a] Numbering according to sequences in GenBank Accession Nos. K03455 (HIV-1), M62321 (HCV 1a), X70185 (HBV adw2), and M10098 (human 18S rRNA).

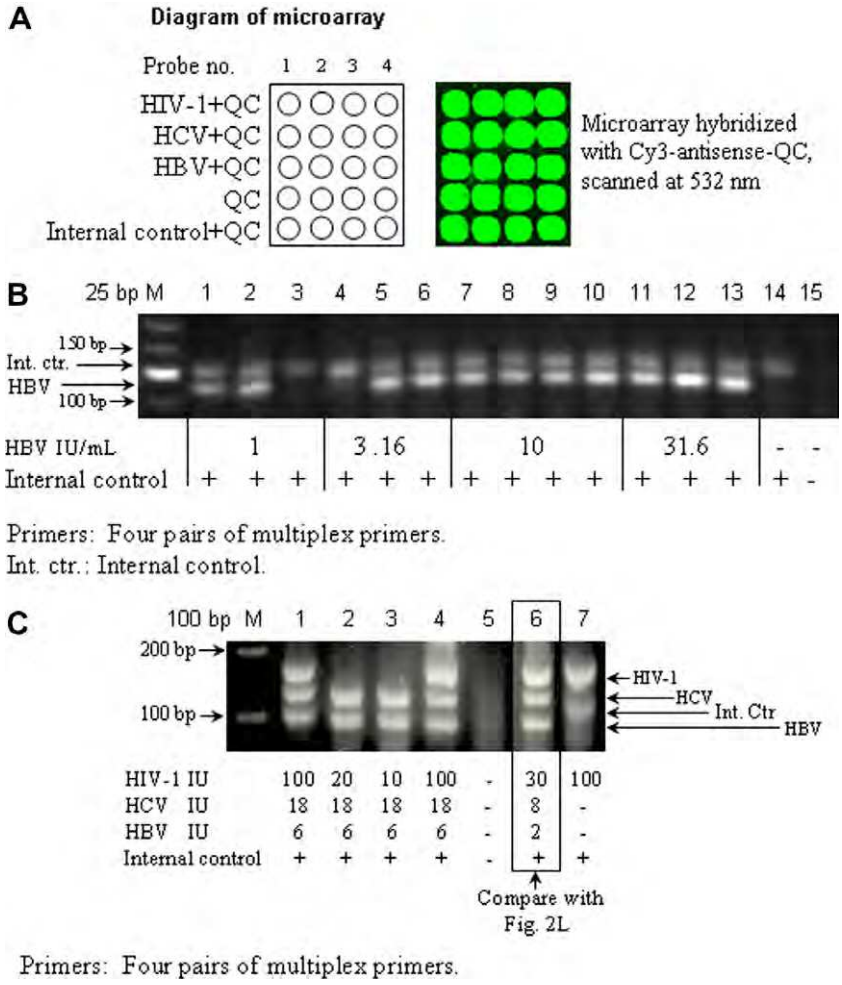

Fig. 1. (A) Diagram of the microarray (left side). Each row (1–5) was spotted with 4 probes of each virus (HIV-1, HCV, HBV) or internal control together with quality control probe (QC), except the 4th row, where only QC was spotted. Quality control test of the microarray (right side). The green signals indicate presence of QC, the figure indicates that all probes were printed evenly on the array. (B) One of the agarose gel electrophoresis analysis (2.5% agarose gels premixed with GelStar stain) of multiplex 2nd PCR products for determination of LOD of HBV DNA. HBV is negative on lanes 3 and 4, both PCR products are positive for HBV on microarrays. Same PCR product was used in lane 3 and Fig. 2B. (C) Simultaneous detection of HIV-1, HCV and HBV nucleic acids by multiplex PCR on gel electrophoresis. In lane 6, the internal control shows as negative, the same PCR product gave positive signals for all the three viruses and the internal control on microarray (Fig. 2L). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

GenBank of known genotypes of HBV using MacVector 7.2 software. HBV genotypes were assigned according to the specificity of genotype-specific amino acids 45, 46, 47, 49, 56, 57, 59, 64, and 85 in the S gene [16,17].

Real-time quantitative PCR. Quantitative PCR using SYBR Green I was used to assess the HBV DNA copy number in all the HBV samples. A standard curve was constructed using 10-fold serial dilutions of pAM-6, a plasmid containing the full genome of HBV subtype adw, genotype A (ATCC, Rockville, MD) [17]. PCR primers Taq-S-11(F) (5′-CTGTGCCTTGGGTGGCTTT-3) and Taq-S-12(R) (5′-AAGGAAAGAAGTCAGAAGGCAAAA-3′) from the conserved region of the S gene of HBV were used to amplify a 91 bp HBV DNA fragment. The SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) with primers (300 nM each) and the DNA sample was amplified on an ABI Prism 7700 Sequence Detector according to manufacturer's protocol, finally followed by a dissociation-curve thermocycle (Applied Biosystems). Copy numbers of unknown samples were calculated using the standard curves generated by Sequence Detector v.1.6.3. software (Applied Biosystems). The specificity of the amplified samples was confirmed by analysis of T_m dissociation curves.

Microarray multiplex assay sensitivity. Based on the data obtained from quantification of HBV samples by real-time PCR, and the known concentration of HCV and HIV-1 samples, multiplex PCR assays were run in triplicate containing 1–100 IU/reaction of HBV DNA, HCV, and/or HIV-1 RNA. All the 2nd PCR products were analyzed by agarose gel electrophoresis, and samples with the lowest amplicon concentrations that produced detectable or undetectable bands in the agarose gels were labeled with Cy5 dCTP and tested in the microarrays.

Lower limit of detection and statistic analysis. The WHO HBV International Standard containing 10^6 IU/mL was used to determine the lower limit of detection (LOD) for the HBV DNA. Multiple replicates of dilutions of the WHO HBV International Standard containing 1 IU/mL, 3.16 IU/mL, 10 IU/mL, and 31.6 IU/mL in normal human plasma spiked with 5 ng of human DNA were extracted with NucliSens reagents. After PCR amplification, the 2nd PCR products from all samples were analyzed by both agarose gel electrophoresis and microarray assays. Probit analysis was used to calculate the 95% and 50% lower limit of detection for HBV by microarray assay and gel electrophoresis, respectively, using SAS software.

Table 2
Viral specific, internal control, and quality control oligonucleotide probes of microarray

Target	Probe No.	Sequence (5′–3′)	Gene	Positions (nt)	T_m (°C)
HIV	1	AACTCATTTGCACCACTGCTGTGCCTTGGAATGCT		8026–8060	68
	2	GCTAGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATCAC		8058–8099	65
	3	ACGACCTGGATGGAGTGGGACAGAGAAATTAACAA	gp[41]	8100–8134	65
	4	GAAATTAACAATTACACAAGCTTAATACACTCCTTAATTGAAGAATC		8124–8170	63
HCV	1	TGCAGCCTCCAGGACCCCCCCTCCCGGGAGA		106–136	73
	2	CTCCCGGGAGAGCCATAGTGGTCTGCGGAACCG		126–158	68
	3	GAACCGGTGAGTACACCGGAATTGCCAGGACGACC	5′-UTR	153–187	68
	4	GGACGACCGGGTCCTTTCTTGGATCAACCCGCTC		180–213	71
HBV	1	AATCACTCACCAACCTCCTGTCCTCCAATTTGTCC		329–363	65
	2	TGTCCTGGTTATCGCTGGATGTGTCTGCGG	S-gene	359–388	66
	3	GTGTCTGCGGCGTTTTATCATATTCCTCTTCA		379–410	64
	4[a]	CAGTCCCCAACCTCCAATCACTCACCAACCTCC		314–346	65
Internal Control	1	GTCGTAGTTCCGACCATAAACGATGCCGACCGG		1166–1198	67
	2	GGCGATGCGGCGGCGTTATTCCCATGACCC	18S rRNA	1197–1226	69
	3	CCGCCGGGCAGCTTCCGGGAAACCAAAGTCTTTG		1225–1258	71
	4[a]	TCGAAGACGATCAGATACCGTCGTAGTTCCGACC		1147–1180	67
Quality control probe (QC)		TTGGCAGAAGCTATGAAACGATATGGG	N.A.		82
Antisense-QC		CCCATATCGTTTCATAGCTTCTGCCAA	N.A.		82

[a] The probes No. 4 of HBV and internal control are anti-primer probes as additional controls indicating the presence of primers in reaction mix.

Results

Genotypes and copy number of HBV DNA of HBV samples

The genotype determination of the 19 HBV samples showed that 10 samples were genotype A including the WHO International Standard for HBV DNA NAT, two samples were genotype B, four were genotype C, two samples were genotype D, and one sample was genotype E. The standard curve using known amounts of a plasmid containing the whole HBV genome showed a linear correlation (correlation coefficient of 0.997) between the copy number of the plasmid and the threshold cycle (C_T). The quantification of WHO Internatinal Standard for HBV NAT (contains 1×10^6 IU/mL) using our real-time PCR system yielded 5.4×10^6 gEq/mL (1 IU is equivalent to 5 gEq). The HBV copy number determined by real-time PCR of the other 15 HBsAg positive HBV samples varied from 9.3×10^2 to 2.3×10^9 gEq/mL (equivalent to 1.86×10^2 to 4.6×10^8 IU/mL). The three HBsAg-negative HBV samples contained 2.2×10^2 to 3.5×10^3 gEq/mL of HBV DNA (equivalent to 4.4×10^1 to 7×10^2 IU/mL).

Sensitivity and specificity of the microarray multiplex assay

We considered that a sample was ''positive'' if at least one of viral probes and at least one internal probe showed positive signal. Negative plasma samples did not result in detectable signal on any of viral probes in the microarray, but the internal control probes show positive signals (Fig. 2A). The microarray multiplex assay detected HBV genotype A–D at a 1 IU/mL (Fig. 2B–E) and genotype E at 2 IU/mL (Fig. 2F). The assay detected HCV (genotype 1a, 1b, 3a) at 10 IU/mL (Fig. 2G–I); and HIV-1 (group M, subtype B) at 20–30 IU (Fig. 2J and K). Furthermore, the multiplex microarray assay detected 2 IU HBV, 8 IU HCV, and 30 IU HIV-1 present simultaneously in one sample (Fig. 2L). The absence of viral specific signal for normal plasma sample (Fig. 2A) and the lack of cross-hybridization between three viruses showed that the assay was highly specific (Fig. 2B–K).

Probit analysis of test results on determining the lower limit detection (LOD) of HBV DNA showed that the microarray test had a 95% and 50% lower limit of detection of 1.1 IU/mL and 0.8 IU/mL for HBV DNA, respectively; while the 95% lower limit of detection by gel analysis was 15.9 IU/mL. The results showed that the combination of multiplex PCR and microarray was approximately 14-fold more sensitive than the multiplex PCR and gel electrophoresis analysis. The increased sensitivity of the microarray analysis was evident in samples that were negative by gel analysis but positive in the microarray analysis (compare Fig. 1B lane 3 with 2B, and Fig. 1C lane 6 with 2L).

Discussion

HBV, HCV, and HIV-1 are major human pathogens transmitted by blood and blood products. Approximately 1–1.25 million HBV chronic carriers live in the US (Vierling, et al., Report of American Liver Foundation, 1998), and about two billion individuals have been infected with this virus worldwide (WHO Fact sheet #204, Oct. 2000). There are about 3.9 million individuals infected with HCV in the US and 170 million (3.5% of world population) worldwide [18]. HBV and HCV are the leading causes of liver cirrhosis and hepatocellular carcinoma. There are an estimated 1–1.2 million HIV infected individuals in the US and 49 million more worldwide (Joint United Nations Program on HIV/AIDS, 2004, Global summary of the

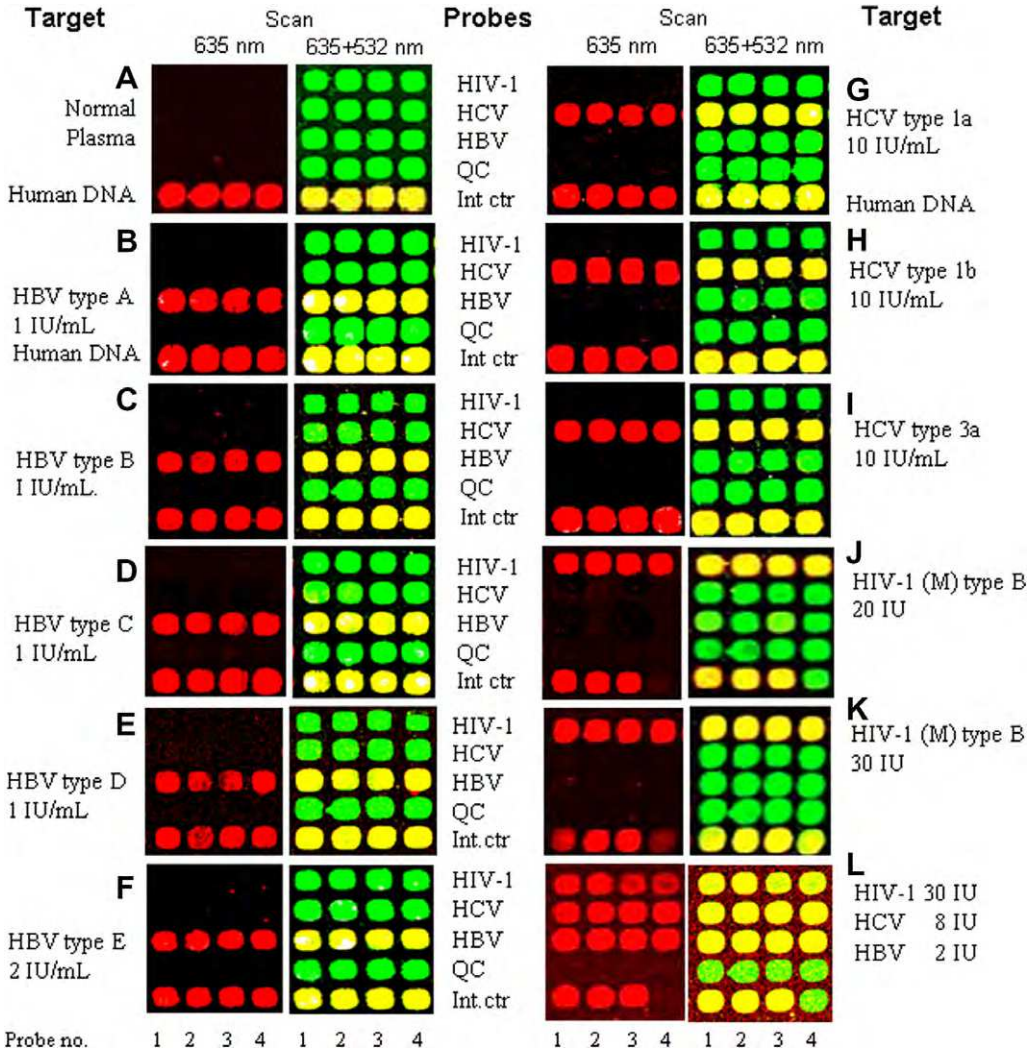

Fig. 2. (A–L) Microarrays were hybridized with different Cy5 labeled gene-specific amplicons plus Cy3-antisencse QC. Each sample has two images. Left image of each sample was scanned at 635 nm wave length laser light, red spots showed the positive hybridization of Cy5-labelled viral and internal control amplicons with the signature probes on the arrays. Right image of each sample was scanned at 635 + 532 nm dual wave lengths, green spots represent the hybridization of Cy3-antisense-QC with QC probes on array; yellow spots demonstrates the simultaneous hybridizations of Cy5-gene-specific amplicons and Cy3-antisense-QC to the probes on array. All multiplex reactions were done in the presence of human DNA as the internal control. (A) Normal plasma (negative control), only Cy5-labeled amplicons of 18S rRNA gene hybridized with the internal control probes on the array. (B) WHO International Standard for HBV NAT (genotype A) at 1 International Unit (IU)/mL, HBV in this sample was negative in the gel electrophoresis analysis (Fig. 1B, lane 3). (C–E) HBV samples of genotype B-D at 1 IU/mL. (F) HBV of genotype E at 2 IU/mL. In Fig. 2B–F, only HBV and internal control probes show signals, no cross reaction with HCV or HIV-1 was observed. (G–I) WHO International Standard for HCV NAT (genotype 1a), CBER HCV RNA panel (genotype 1b) and NIBSC HCV working reagent (genotype 3a), respectively, at 10 IU/mL. Only HCV and internal control probes show positive signals. (J and K) Samples of HIV-1 (group M, subtype B) at 20 and 30 IU/reaction, respectively. (L) Simultaneously detection of HIV-1 (30 IU, group M, subtype B), HCV (8 IU, genotype 1b) and HBV (2 IU, genotype A) in one sample, the internal control is undetectable on gel (Fig. 1C, lane 6) but it is positive on microarray.

AIDS epidemic, WHO). Control of these three viral infections has been a major global health issue.

Transmission of HBV, HCV, and HIV-1 occurs primarily through exposure to infected blood. Since the introduction of serological tests for HBV, HCV, and HIV-1, and implementation of NAT assays for HCV and HIV-1, the risk of transmission by blood transfusions or blood products decreased dramatically. However, there is still a small but significant transfusion transmission risk [19,20]. Currently, there are several commercial NAT systems that used different amplification methods, including PCR, transcription-mediated amplification (TMA), ligase chain reaction, and branched DNA (bDNA) signal amplification assay. Most commercial NAT assays detect only one of these three viruses individually. There are two FDA-licensed duplex NAT assays for blood donor screening capable of detecting both HCV and HIV-1 at a sensitivity of 100 copies/mL for each virus [3]. Multiplex assay for HBV, HCV, and HIV for donor screening is under development. A reported automated multiplex system using TaqMan PCR for the simultaneous detection of these three viruses had a detection limit of 22–60 copies/mL (equivalent to about 4.4–12 IU/mL) for HBV, 61–112 IU/mL for HCV, and 33–66 copies/mL for HIV-1 (I IU equivalent to 0.65–

1 copies) [4]. But these duplex or multiplex assays require further confirmation tests to discriminate and identify the specific virus or viruses. Our microarray assay uses 3–4 probes per viral marker, therefore has the ability to detect a wider range of genotypes and viral variants arising in the field. Indeed, this microarray assay detected HBV genotype A–E (accounts for 98% of HBV-positive cases in US source plasma donors), HCV type 1a, 1b, and 3a (accounts for 81% of chronic HCV in the US), and HIV-1 group M, subtype B (accounts for 95% of HIV infections in the US). The primers we designed actually matched the respective sequences of all the common genotypes of each of these three viruses. The microarray assay described in this paper could be used in the research to study infections with these viruses in any kind of human or animal (for example, chimpanzees) samples. This assay seems especially suitable for studies in high-risk patients who have high co-infection rates of HIV-1 and HCV or HIV-1 and HBV. Our microarray assay is also suitable as confirmation test in donor screening, currently confirmation tests were done by three NAT assays for HBV, HCV, and HIV-1. It will be cost-effective if one microarray multiplex assay could replace these three individual NAT assays. Our studies showed that the 95% LOD of HBV by our microarray assay is 1.1 IU/mL, which is more sensitive than currently reported NAT assays, and is approximately 14-fold more sensitive than gel electrophoresis analysis. Other multiplex assays such as a real-time PCR [21], a flow cytometer microsphere-based assay [22], and a visual DNA chip [23] had higher LODs. For instance, the real-time PCR using multiple fluorescent probes had a 95% detection limit of 30, 167, and 680 IU/mL for HBV DNA, HCV RNA, and HIV-1 RNA [21], respectively. The dipstick assay had a detection limit of 50, 125, and 500 IU/mL for HBV DNA, HCV RNA, and HIV-1 RNA, respectively [24].

Our study constitutes a proof-of-concept for the use of microarray technology in the simultaneous detection of multiple human pathogens. Further research will be required to improve, simplify and automate this microarray assay.

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Basic Concepts of Microarrays and Potential Applications in Clinical Microbiology

Melissa B. Miller[1]* and Yi-Wei Tang[2]

Department of Pathology and Laboratory Medicine, School of Medicine, University of North Carolina, Chapel Hill, North Carolina,[1] and Departments of Medicine and Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee[2]

INTRODUCTION 611
BASIC CONCEPTS OF MICROARRAYS 611
Printed Microarrays 612
In Situ-Synthesized Oligonucleotide Microarrays 615
High-Density Bead Arrays 616
Electronic Microarrays 617
Suspension Bead Arrays 618
POTENTIAL APPLICATIONS IN CLINICAL MICROBIOLOGY 621
Microbial Detection and Identification 621
Respiratory Viral Pathogen Detection in Connection with Multiplex PCR Amplification 622
Simultaneous Detection and Typing of Human Papillomaviruses 623
Rapid Detection and Characterization of Methicillin-Resistant *Staphylococcus aureus* 624
Determination of Antimicrobial Drug Resistance 624
Microbial Typing 624
Microbial Gene Expression Profiling 625
Host Gene Expression Profiling during Microbial Infections 625
Host Genomic Polymorphism Determination 625
CONCLUDING REMARKS 626
REFERENCES 627

INTRODUCTION

Molecular detection techniques continue to increase in utility in clinical microbiology laboratories. The implementation of in vitro nucleic acid amplification techniques, led by real-time PCR, in diagnostic laboratories has transformed viral detection and select bacterial detection. Although not likely to completely replace culture techniques in the near future, molecular applications in the diagnosis of infectious diseases have become commonplace in academic medical centers and tertiary-care facilities and are becoming more tangible in community-based settings as more FDA-cleared products are available. The further advancement of molecular infectious disease diagnostics is dependent on the ability of multiplexing technologies, or the ability to detect and identify more than one pathogen simultaneously from the same specimen, to be implemented in clinical microbiology laboratories with ease and accuracy. One approach to multiplex detection and characterization is microarray analysis.

Simply defined, a microarray is a collection of microscopic features (most commonly DNA) which can be probed with target molecules to produce either quantitative (gene expression) or qualitative (diagnostic) data. Although other types of microarrays exist, such as protein microarrays (122, 125), this review will focus on DNA microarrays. The initial production of arrays in the research arena included radiolabeled macroarrays such as Southern blots and dot blots (91, 177). Scientific ingenuity in research laboratories in the 1990s led to the development of modern two-dimensional hybridization microarrays (167, 172). Largely due to advances in fabrication, robotics, and bioinformatics, microarray technology has continued to improve in terms of efficiency, discriminatory power, reproducibility, sensitivity, and specificity (135). In addition, microarray platforms have expanded to include three-dimensional arrays or suspension bead arrays. These improvements have allowed the transition of microarrays from strictly research settings to clinical diagnostic applications. The number of articles on microarrays and articles describing their use in microbiology and infectious diseases has rapidly increased over the past 9 years (Fig. 1). Although many of these articles can still be attributed to clinical microbiology or infectious disease "research," research in the diagnostic realm has led to the optimization of the diagnostic potential of microarrays and has led to the development of commercially available qualitative detection platforms. Thus, we have now entered a new era in molecular diagnostics where the use of microarray technology in clinical microbiology is a reality.

BASIC CONCEPTS OF MICROARRAYS

Microarrays can be distinguished based upon characteristics such as the nature of the probe, the solid-surface support used, and the specific method used for probe addressing and/or target detection (135). Below, we review the methodologies of printed and in situ-synthesized microarrays, high-density bead arrays, and electronic and suspension bead microarrays. In all

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, UNC School of Medicine, Campus Box 7525, Chapel Hill, NC 27599-7525. Phone: (919) 966-3723. Fax: (919) 966-0486. E-mail: mbmiller@unch.unc.edu.

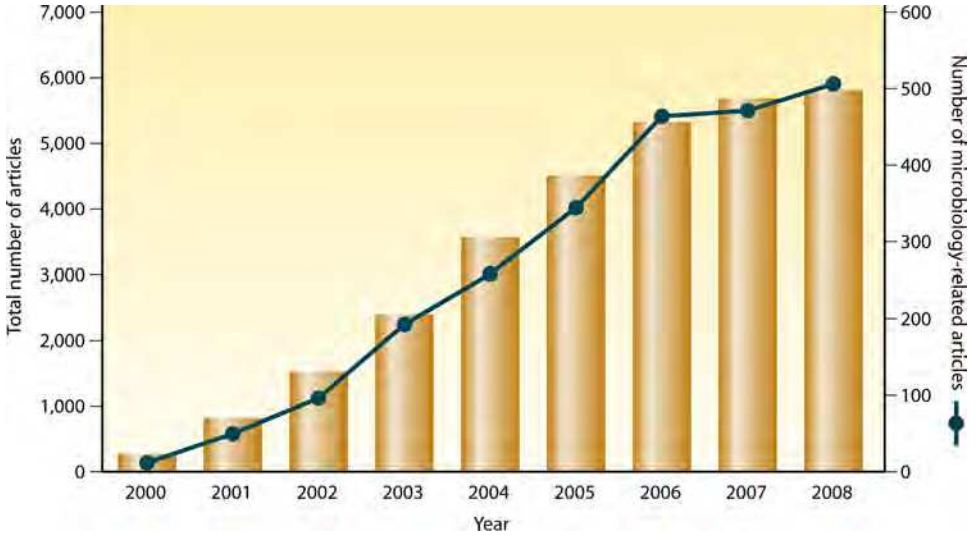

FIG. 1. Microarray publications. The number of primary manuscripts published using microarray technology (bars) and the number of microarray publications that have infectious disease and/or microbiology applications (line) are depicted.

of these approaches, the probe refers to the DNA sequence bound to the solid-surface support in the microarray, whereas the target is the "unknown" sequence of interest. In general terms, probes are synthesized and immobilized as discrete features, or spots. Each feature contains millions of identical probes. The target is fluorescently labeled and then hybridized to the probe microarray. A successful hybridization event between the labeled target and the immobilized probe will result in an increase of fluorescence intensity over a background level, which can be measured using a fluorescent scanner (135). The fluorescence data can then be analyzed by a variety of methods. Experimental details including probe length and synthesis, number of possible features (i.e., density of the microarray), and the solid surface used vary depending on the type of microarray employed and are discussed below and summarized in Table 1. The goals of the manuscript are to review the concepts behind each of these microarray technologies, highlighting their benefits and disadvantages, as well as provide a detailed review of the applications of these techniques in clinical microbiology.

Printed Microarrays

Printed arrays were the first microarrays utilized in research laboratories and are so called because of the "printing" or spotting of the probes onto the microarray surface, which is most commonly a glass microscope slide. Glass slides are an attractive medium for microarrays because they are economical; are stable throughout high temperatures and stringent washes; are nonporous, allowing for efficient kinetics during hybridization; and have minimal background fluorescence (25). The probe spots, or features, can be applied by either noncontact or contact printing. A noncontact printer uses the same technology as computer printers (i.e., bubble jet or inkjet) to expel small droplets of probe solution onto the glass slide. In contact printing, each print pin directly applies the probe solution onto the microarray surface. The result in both cases is the application of a few nanoliters of probe solution per spot to create an array of 100- to 150-μm features. During the printing process it is imperative to control for cross-contamination and printing consistency to preserve the integrity of the microarray and subsequent hybridization data. Due to the relatively large size of the features, printed microarrays are of lower density (~10,000 to 30,000 features) than in situ-synthesized microarrays and high-density bead arrays (discussed below) but offer considerably more features than either electronic microarrays or suspension bead arrays. The general workflow for the processing of printed microarrays is depicted in Fig. 2.

Printed arrays can be further classified based upon the nature of the probes: double-stranded DNA (dsDNA) or oligonucleotide microarrays. For dsDNA microarrays, the probes consist of amplification products (amplicons) obtained by PCR using primers designed from a known genomic sequence, shotgun library clones, or cDNA (74, 167, 194). The double-stranded amplicons are denatured, either in print buffer or after immobilization, which allows the probes to be available for hybridization. Amplicons can be attached to the glass slide surface by the electrostatic interaction of the negative charge of the phosphate backbone of the DNA with a positively charged coating of the slide surface (51) or by UV-cross-linked covalent bonds between the thymidine bases in the DNA and amine groups on treated slides (25). Typically, each 200- to 800-bp dsDNA probe represents a different gene. Ideally, PCR amplicons for microarrays should have high specificity and yield but no contamination, including nonspecific amplification and contaminants that affect attachment to the microarray surface or that autofluoresce (19). Unfortunately, dsDNA probes generally have a high sensitivity but suffer in specificity. For example, in a report by Hager, 21 to 34% of probes did not match the intended target and/or were contaminated (72). The ultimate assessment of probe specificity is sequencing of the products. However, due to financial constraints, most laboratories test the purity and quantity of the amplified product by agarose gel electrophoresis. The specificity of hybridization

TABLE 1. Comparison of microarray platforms[a]

Microarray	Principle(s)	Format(s)	Density	Relative cost	Diagnostic application(s)	References
Printed	Glass slides are used as the solid support for printing DNA probes	For dsDNA, PCR amplicons (200–800 bp) from known genomic sequence, shotgun library clones, or cDNA are used; for oligonucleotides, 25-80-bp probes are synthesized	Moderate (~10,000–30,000)	$$$	No commercially available applications; pathogen detection and identification, antimicrobial resistance detection, viral discovery, molecular surveillance	21, 32, 153, 167, 172, 195, 206–208
In situ synthesized	Oligonucleotides are synthesized directly on the surface of a quartz wafer using photochemistry; multiple probe sets (one perfect-match probe and one mismatch probe) are included per target	Affymetrix GeneChips, 20-25-bp probes; Roche NimbleGen, 60-100-bp probes; Agilent, 60-bp probes	High (Affymetrix, $>10^6$; NimbleGen and Agilent, 15,000–$>10^6$)	$$$$	No commercially available applications; pathogen detection and identification, antimicrobial resistance detection, viral discovery, molecular surveillance, strain typing	59, 117, 150, 151, 157, 200, 215
High-density bead arrays	Sequence-tagged beads are randomly assorted onto fiber-optic bundles or silicon slides	SAM, 96 samples; Sentrix BeadChip; 1-16 samples	High (~50,000-10^6)	$$$	No commercially available applications; potential use in microbiology but no studies published to date	52, 71, 146
Electronic	Electric fields are used to promote active hybridization of nucleic acids on a microelectronic device; streptavidin-biotin bonds immobilize the probes on the array surface	NanoChip 400; capture probe down; amplicon down; sandwich assays	Low (400 max)	$$	Commercially available products discontinued; pathogen detection and identification	10, 108, 185, 226
Liquid-bead suspension	Spectrally unique microspheres provide solid support for application of probes or universal sequence tags; bead hybridization with fluorescently labeled target DNA is measured using flow cytometry	Direct DNA hybridization; competitive DNA hybridization; solution-based chemistries (ASPE/TSPE, OLA, SBCE)	Low (100 max)	$$	FDA-cleared xTAG RVP assay; pathogen detection and identification, antimicrobial resistance detection, strain typing	18, 73, 79, 109, 112, 148, 191

[a] Data from reference 135. $$, low-moderate cost; $$$, moderate cost; $$$$, high cost.

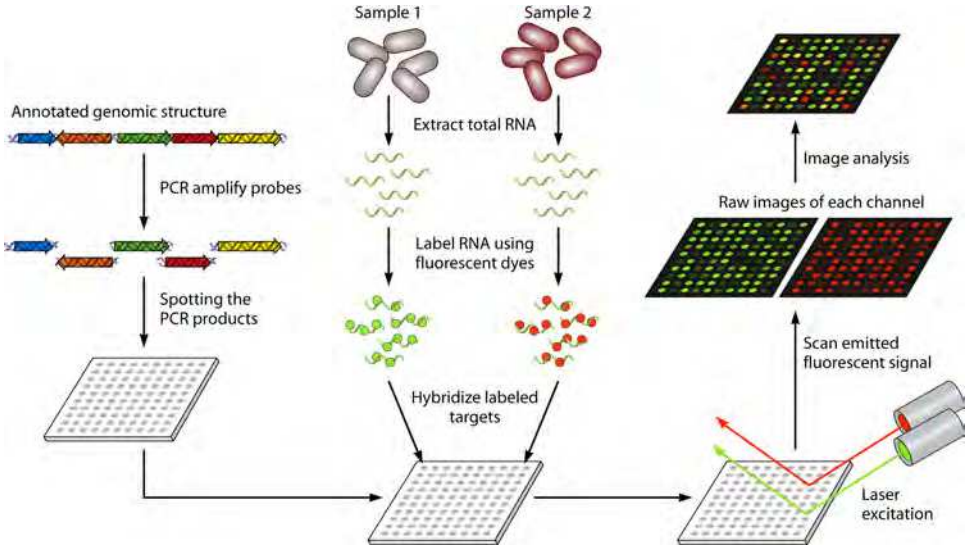

FIG. 2. Workflow summary of printed microarrays. Probes are PCR amplified (or oligonucleotides are synthesized) and subsequently spotted onto a glass slide. In this example, two samples to be compared undergo RNA extraction, cDNA production, and differential fluorescent labeling. Hybridization of labeled target nucleic acids to the probe array allows fluorescent scanning to provide data for analysis. (Adapted from reference 51 [Fig. 1A, © Springer-Verlag 2006] with kind permission from Springer Science and Business Media.)

data can be improved by incorporating redundancy by the inclusion of multiple gene segments among the probes. Although decreased specificity can be beneficial when analyzing a genomic sequence rich in natural polymorphisms, it is disadvantageous when trying to discriminate among highly similar target sequences and unacceptable for clinical diagnostic applications (105).

The spotted probes of oligonucleotide microarrays consist of short, chemically synthesized sequences. In contrast to the relatively large size of dsDNA probes discussed above, the length of probes typically used in printed oligonucleotide microarrays ranges from 25 to 80 bp but may be as long as 150 bp for gene expression microarrays (30). The use of shorter probe lengths allows fewer errors to be introduced during probe synthesis and facilitates the interrogation of small genomic regions, including polymorphisms. While the decreased probe length may adversely affect sensitivity compared to dsDNA probes, specificity is often greater when short, specific genomic regions are interrogated. Longer probes have higher melting temperatures and greater mismatch tolerance, leading to decreased specificity. Generally, the strength of the hybridization signal and the sensitivity increase with an increasing length of the probe. The addition of spacers coupled to very short oligonucleotides or the application of a higher concentration of probe during printing can improve the hybridization signal strength (30). Ramdas et al. reported an eightfold increase in sensitivity with 70-mer oligonucleotides relative to the sensitivity of 30-mer probes, especially for low-expression genes (159). Since, as with dsDNA probes, very long oligonucleotide probes may decrease specificity due to random hybridization to nontarget sequences, it is critical to cautiously determine the optimal probe length for each microarray design. Although easier to manufacture than dsDNA probes, oligonucleotide probes require careful design so that all probes have comparable melting temperatures (within 5°C) and lack palindromic sequences. Preferably, each probe should be tested experimentally to guarantee nonbiased hybridization data, although this may not always be financially possible (30). Oligonucleotide probes are attached to glass slides by covalent linkage because, due to their small size, a significant quantity of probes would be lost during wash steps after noncovalent electrostatic immobilization and cross-linking. The probes are coupled to the microarray surface via modified 5′ or 3′ ends (most commonly a 5′ amino group) on coated slides that provide aldehyde or epoxy functional groups.

Compared to the in situ-synthesized microarrays discussed below, printed microarrays are relatively simple and inexpensive. However, the initial setup of microarray facilities is costly and requires dedicated space in which environmental variables such as dust, humidity, and temperature are well controlled. Dedicated microarray core facilities are available at many universities, making these challenges minimal for individual investigators. A major advantage of printed microarrays is flexibility. The ability to quickly adjust spotted probes based upon updated annotations or the discovery of new, emerging pathogens or resistance mechanisms makes printed microarrays attractive for use in clinical microbiology. However, the implementation of a printed microarray in a clinical diagnostic laboratory is complicated by the arduous and expensive tasks of monitoring production reproducibility, performing clinical validation studies, and continuously assessing the quality of downstream data. A major drawback in the manufacturing of printed dsDNA microarrays is the enormous scale of amplicon production and the associated difficulties of quality control, information management, efficiency, and accuracy. Likewise, the design of oligonucleotide probes is labor-intensive, and errors introduced from probe synthesis are a problem. Although printed microarrays are conducive to "homebrew" or user-defined testing, their use in diagnostic microbiology remains limited to specific research applications. Printed dsDNA

microarrays are also crucial to the study of organisms that have not been fully sequenced. The lack of commercially available printed microarrays for use in clinical infectious disease diagnostics makes it unlikely that printed microarrays will soon transition to clinical microbiology laboratories. There are, however, commercially available whole-genome microarrays for select organisms that are useful for research endeavors.

In Situ-Synthesized Oligonucleotide Microarrays

In situ-synthesized arrays are extremely-high-density microarrays that use oligonucleotide probes, of which GeneChips (Affymetrix, Santa Clara, CA) are the most widely known. Unlike the printed oligonucleotide arrays described above, the oligonucleotide probes are synthesized directly on the surface of the microarray, which is typically a 1.2-cm^2 quartz wafer. Because in situ-synthesized probes are typically short (20 to 25 bp), multiple probes per target are included to improve sensitivity, specificity, and statistical accuracy. Classically, 11 probes are used per 600 bases being examined (38). The use of probe sets further increases the specificity. A probe set includes one perfect-match probe and one mismatch probe that contains a 1-bp difference in the middle position of the probe (i.e., position 13 of a 25-bp probe). Each member of the probe set is located in a separate feature, which allows the mismatch probe to act as a negative control to identify possible nonspecific cross-hybridization events. Recent advances in GeneChips include the use of longer probes, the design of arrays that interrogate across entire genes or exons, and the implementation of multiple independent and nonoverlapping perfect-match probes in lieu of classic probe sets.

Affymetrix GeneChips typically have $>10^6$ features per microarray depending on the interfeature distance (38, 59). GeneChip oligonucleotide probes are synthesized using semiconductor-based photochemical synthesis. On the quartz surface are synthesis linkers modified with light-sensitive protecting groups (59). Thus, the microarray surface is chemically protected from a nucleotide addition until deprotected by light. When the array surface is exposed to UV light, reactive nucleotides modified with a photolabile protecting group can be added to growing oligonucleotide chains. To target specific nucleotides to exact probe sites, photolithographic masks are used. Each photolithographic mask has a defined pattern of windows, which acts as a filter to either transmit or block UV light from specific features on the chemically protected microarray surface. Areas of the microarray surface in which UV light has been blocked will remain protected from the addition of nucleotides, whereas areas exposed to light will be deprotected, and specific nucleotides can be added. The pattern of windows in each mask directs the order of nucleotide addition. In situ probe synthesis is therefore accomplished through the cycling of masking, light exposure, and the addition of either A, C, T, or G bases to the growing oligonucleotide (Fig. 3) (38, 51).

Other high-density oligonucleotide microarrays include those manufactured by Roche NimbleGen (Madison, WI) and Agilent Technologies (Palo Alto, CA). Both platforms use longer oligonucleotide probes (60 to 100 bp), but NimbleGen uses maskless photo-mediated synthesis, and Agilent employs inkjet technology for the in situ manufacturing of the probes. While experiments performed with GeneChips are limited to one label, the NimbleGen and Agilent platforms allow multicolor hybridizations. As mentioned above for printed microarrays, the use of longer oligonucleotides increases sensitivity. The Roche NimbleGen approach to in situ synthesis is similar to that of the GeneChips described above, but photolithographic masks are replaced by "virtual" or digital masks in Roche NimbleGen's maskless array synthesizer technology. Maskless array synthesizer technology uses an array of programmable micromirrors to create digital masks that reflect the desired pattern of UV light to deprotect the features where the next nucleotide will be coupled (Fig. 4). Each NimbleGen microarray can contain $>10^6$ features and can be purchased in the following formats per slide: 1×2.1 million features, $3 \times 720{,}000$ features, $1 \times 385{,}000$ features, $4 \times 72{,}000$ features, and $12 \times 135{,}000$ features. In contrast to the quartz wafers used for the above-described technologies, Agilent microarrays use glass slides and inkjet printing, which eliminates the need for either lithographic or digital masks (Fig. 5). The in situ synthesis of 60-mer oligonucleotides is achieved using five-"ink" (4 bases plus catalyst) printing of nucleotide precursors combined with coupling and deprotection steps (83). Agilent microarrays are available in the following formats: $1 \times 244{,}000$ features, $2 \times 105{,}000$ features, $4 \times 44{,}000$ features, and $8 \times 15{,}000$ features.

Due to the complex nature of chemical synthesis and the expense involved in production, synthesized microarrays are dependent on commercial manufacturing and are therefore not conducive to user-defined development. There is a growing number of microbial genome microarrays available commercially for gene expression studies. Also, several resequencing microarrays have been developed by TessArae (Potomac Falls, VA) on the Affymetrix GeneChip platform to simultaneously detect and differentiate large numbers of microbial pathogens (115, 116). Currently, a synthesized oligonucleotide array appropriate for use in a diagnostic microbiology laboratory would need to be ordered as a custom microarray. The expense of a custom Affymetrix microarray and the inherent inflexibility of its custom mask make the use of an Affymetrix-synthesized array impractical for the clinical laboratory. In contrast, other in situ-synthesized platforms (Nimblegen and Agilent) can be easily customized with unique oligonucleotide sequence content. Furthermore, a Web-based tool provided by Agilent (eArray) allows users to custom design microarrays with no minimum manufacturing batch size, making Agilent microarrays a primary choice for homebrew and pilot applications. Although it is relatively expensive to obtain a custom oligonucleotide array, many universities already have one or more of these platforms on campus for hybridization and analysis, which may offset the upfront costs. The major advantages to these systems are the reproducibility of the manufacturing process and the standardization of reagents, instrumentation, and data analysis, all of which are critical for methodologies to transition to the clinical laboratory (38). Further advantages that make this approach attractive for clinical diagnostics include controls, such as reference probes for intensity normalization; internal standards of known concentrations; and probes arranged in a checkerboard pattern that are homologous to an internal control included in the hybridization mix.

Whether printed or synthesized, oligonucleotide microar-

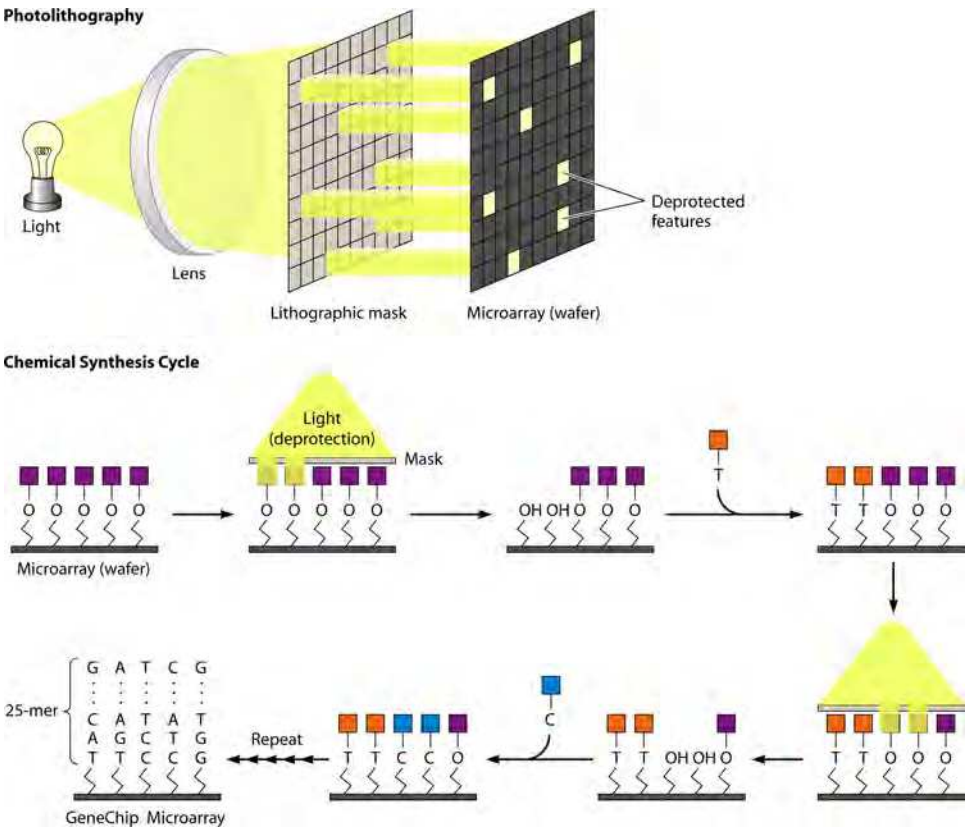

FIG. 3. Affymetrix GeneChip oligonucleotide microarray. (Top) Photolithography. UV light is passed through a lithographic mask that acts as a filter to either transmit or block the light from the chemically protected microarray surface (wafer). The sequential application of specific lithographic masks determines the order of sequence synthesis on the wafer surface. (Bottom) Chemical synthesis cycle. UV light removes the protecting groups (squares) from the array surface, allowing the addition of a single protected nucleotide as it is washed over the microarray. Sequential rounds of light deprotection, changes in the filtering patterns of the masks, and single nucleotide additions form microarray features with specific 25-bp probes. (Adapted from reference 38 with permission of the publisher [copyright Elsevier Inc. 2006].)

rays generally allow much cleaner downstream hybridization data than do amplicon-based microarrays. With oligonucleotide arrays, the ability to standardize probe concentrations and hybridization temperatures while avoiding or controlling for significant nonspecific hybridization has resulted in considerable improvements in the accuracy and reproducibility of microarray data (105). Although in situ-synthesized oligonucleotide microarrays are very robust systems and have significant control measures included, there are currently none with direct diagnostic infectious disease applications that are commercially available.

High-Density Bead Arrays

Similar to the printed and in situ-hybridized microarrays discussed above, BeadArrays (Illumina, San Diego, CA) provide a patterned substrate for the high-density detection of target nucleic acids. However, instead of glass slides or silicon wafers as direct substrates, BeadArrays rely on 3-μm silica beads that randomly self-assemble onto one of two available substrates: the Sentrix Array Matrix (SAM) or the Sentrix BeadChip (Fig. 6) (52, 146). The SAM contains 96 1.4-mm fiber-optic bundles. Each bundle is an individual array consisting of 50,000 5-μm light-conducting fibers, each of which is chemically etched to create a microwell for a single bead (52). In the universal BeadArray, up to 1,536 bead types (each with a unique capture sequence) assemble onto each fiber bundle, resulting in ~30 beads of each type in the array (146). Each SAM allows the analysis of 96 independent samples. The BeadChip can be used to assay 1 to 16 samples at a time on a silicon slide that has been processed by microelectromechanical systems technology to provide microwells for individual beads (53). BeadChips are more appropriate for very-high-density applications such as whole-genome genotyping, which requires 10^5 to 10^6 features for determining genome-wide single nucleotide polymorphisms (SNP) (Infinium assay; Illumina) (70).

Unlike the known locations of printed and in situ-hybridized microarray features, the beads in BeadArrays randomly assort to their final location on the array. Thus, the bead location must be mapped, which is accomplished by a decoding process (71). This "decoding" process is in contrast to the use of internal dyes for "encoding" the Luminex microspheres discussed in "Suspension Bead Arrays" below. Each bead has ~700,000 copies of a unique capture oligonucleotide covalently attached to it, which serves as the bead's identifier (107). In the universal arrays, the capture sequences specifically avoid homology with human and mouse nucleic acid sequences and are referred to as IllumiCodes. The mapping of

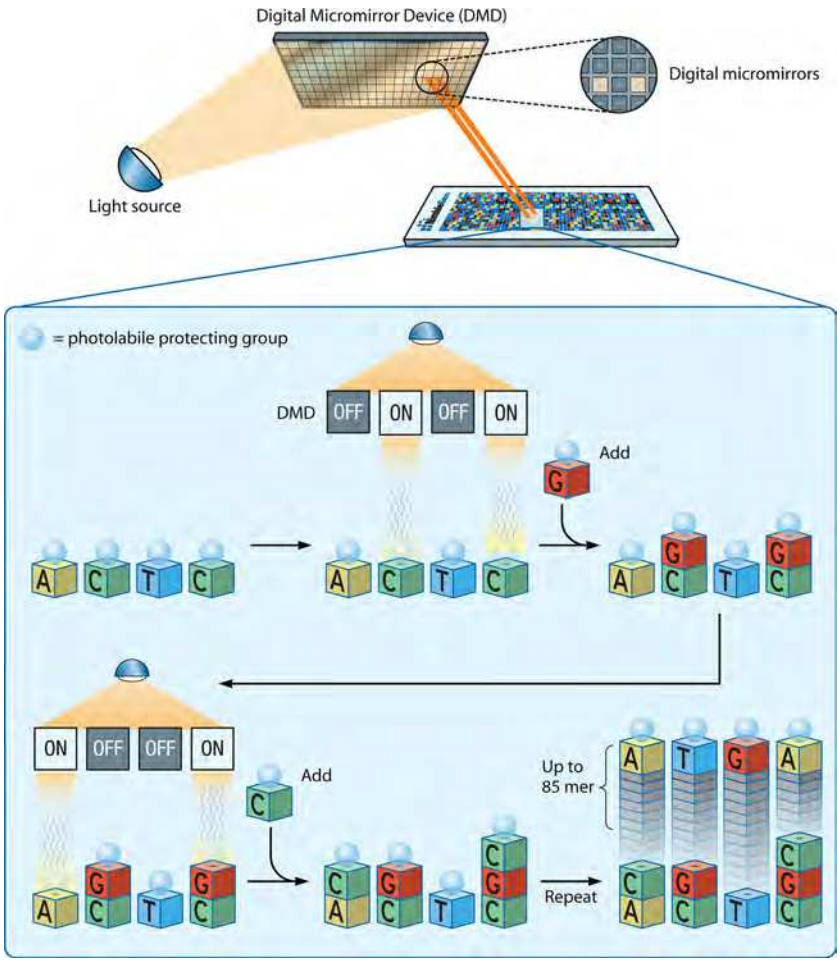

FIG. 4. Roche NimbleGen oligonucleotide microarray. Maskless array synthesizer technology is depicted, which utilizes a digital micromirror device (DMD) to create virtual masks. The DMD forms the pattern of UV light needed to direct the specific nucleic acid addition during photo-mediated synthesis. UV light removes the photolabile protecting group (circles) from the microarray surface, allowing the addition of a single protected nucleotide to the growing oligonucleotide chain. The cycling of DMD filtering, light deprotection, and nucleotide addition creates oligonucleotide features 60 to 100 bp in length on the NimbleGen microarray. (Courtesy of Roche NimbleGen [copyright Roche NimbleGen, Inc.].)

the Illumina beads is accomplished by a series of hybridization and rinse steps, allowing fluorescently labeled complementary oligonucleotides to bind to their specific bead sequence (IlluminiCode) and thus track the location of the bead type (52). An additional advantage to the decoding process is the quality control provided for each feature of the microarray (53).

The SAM can be processed using a standard microtiter plate, which makes it amenable to standard automation and high-throughput processing. The distance between individual arrays on the 16-sample BeadChip is identical to that of a standard multichannel pipettor, thereby facilitating ease of use. BeadArrays can support up to 10^5 to 10^6 features and have built-in redundancy. This redundancy is a crucial experimental control for intermicroarray comparative data since each manufactured microarray will not be identical. An additional advantage to the uniqueness of each microarray is that altering the bead pattern provides a means to identify spatial bias. Although the analysis tools available for BeadArray-specific data analysis, background correction, and spatial artifact recognition have been lagging behind those provided by other microarray manufacturers, independent researchers have begun to fill the gaps (20, 47, 49, 50, 182, 217). BeadArrays have been successfully applied to DNA methylation studies (12, 13), gene expression profiling (14, 54, 107), and SNP genotyping, including the International HapMap Project (www.hapmap.org) (23, 53, 70).

Electronic Microarrays

The printed and in situ-synthesized microarrays and BeadArrays described above rely on passive transport for the hybridization of nucleic acids. In contrast, electronic microarrays utilize active hybridization via electric fields to control nucleic acid transport. Microelectronic cartridges (NanoChip 400; Nanogen, San Diego, CA) use complementary metal oxide semiconductor technology for the electronic addressing of nucleic acids (175). Each NanoChip cartridge has 12 connectors that control 400 individual test sites. Negatively charged nucleic acids are transported to specific sites, or features, when a positive current is applied to one or more test sites on the

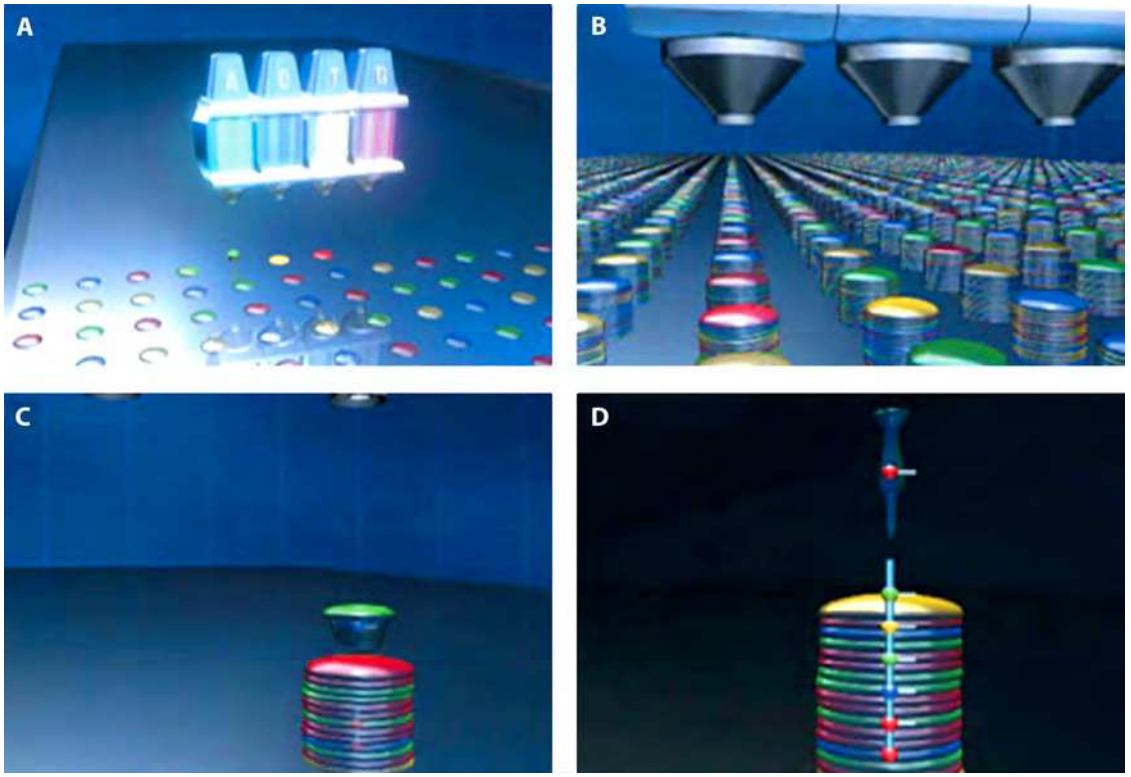

FIG. 5. Agilent oligonucleotide microarray. (A) Noncontact inkjet printing technology delivers a small and accurate volume (picoliters) of nucleotides to the first layer on the microarray surface. (B) Repeated rounds of base-by-base printing extend the length of specific oligonucleotide probes. (C) Close-up of growing oligonucleotide chain with a base being added. (D) The final product is a 60-mer in situ-synthesized probe as a feature on a microarray containing thousands of specifically synthesized probes. (Images courtesy of Agilent Technologies.)

microarray. The surface of the microarray contains streptavidin, which allows for the formation of streptavidin-biotin bonds once electronically addressed biotinylated probes reach their targeted location. The positive current is then removed from the active features, and new test sites can be activated by the targeted application of a positive current. Once the probes have been hybridized at discrete features, the microarray is ready for the application of fluorescently labeled target DNA. Typically, target DNA passively hybridizes with the immobilized probes on the microarray but can also be concentrated electronically (Fig. 7). Although addressing the capture probe down first is the most commonly used format, amplicon-down and sandwich assays have also been utilized. Regardless of the addressing format used, if hybridization occurs between the probe and the target DNA, fluorescent reporters will be present at the positive test, which will be detected when the electronic microarray is scanned and analyzed.

Electronic microarrays offer several advantages. For example, multiplex detection can be accomplished at an individual test site since multiple probes, each with a distinct fluorophore, can be sequentially addressed to the same feature. The flexibility of this platform allows nucleic acids from a single sample to be hybridized to multiple (but not necessarily all) test sites for the detection of multiple targets, or nucleic acids from multiple samples can be analyzed on the same microarray cartridge, minimizing waste. Furthermore, the NanoChip is a universal blank chip, and the content of the microarray is specified directly by the user, which allows more flexibility in assay design and decreases costs associated with microarray manufacturing. Although the density of electronic microarrays is currently limited to 400 spots, this is sufficient for the majority of diagnostic microbiology applications. In 2007, Nanogen announced the termination of its microarray business. Nonetheless, this technology demonstrates the evolution of microarray technology to a platform that is more practical for diagnostic applications.

Suspension Bead Arrays

In contrast to the two-dimensional, or planar, arrays discussed above, suspension bead arrays are essentially three-dimensional arrays based on the use of microscopic polystyrene spheres (microspheres or beads) as the solid support and flow cytometry for bead and target detection. Furthermore, they are distinct from the high-density Illumina BeadArrays discussed above, in which the beads are immobilized on fiber-optic strands or silicon slides. Suspension-bead-based assays were initially described in 1977 and focused on the detection of antigens and antibodies (78). Multiplexing was first achieved by using different-sized microsphere sets for the simultaneous detection of multiple antibodies (130, 169). Currently, more-robust multiplexing is accomplished using different microsphere sets based on color. Red (658-nm emission) and infrared (712-nm emission) fluorochromes are used at various

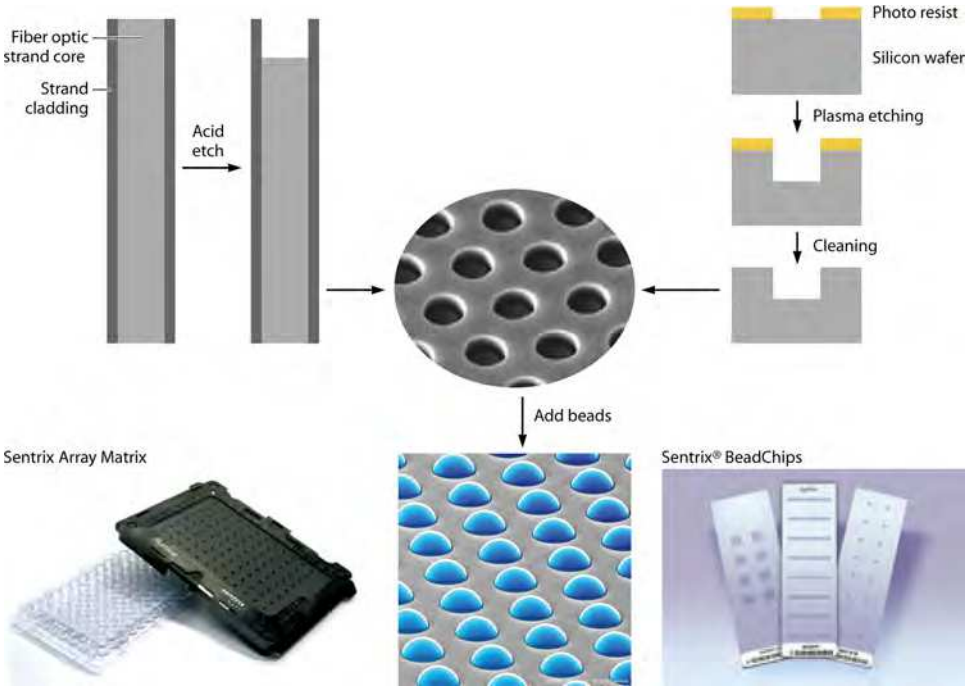

FIG. 6. Illumina BeadArray. The SAM contains 96 1.4-mm fiber-optic bundles (bottom left). Each bundle is an individual array consisting of 50,000 5-μm fiber-optic strands, each of which is chemically etched to create a microwell for a single bead (top left). The Sentrix BeadChips can assay 1 to 16 samples at a time on a silicon slide (bottom right) that has been processed to provide microwells for individual beads (top right). Both BeadArray platforms rely on 3-μm silica beads that randomly self-assemble (center). (Adapted from reference 53 with permission of the publisher. © 2009 BioTechniques.)

concentrations to fill 5.6-μm microspheres. Each bead of the 100-microsphere set has a distinct red-to-infrared ratio, and therefore, each bead has a unique spectral address (Fig. 8A). Microspheres with a specific spectral address coupled to a specific probe are equivalent to a feature in a planar microarray. Once multiple individual microspheres have been coupled to separate specific probes, a mixture of microspheres (in theory, up to 100) can be used to interrogate extracted and amplified nucleic acids (Fig. 8B). The subsequent detection of a fluorescent reporter that indicates probe-target DNA hybridization is accomplished using a bench-top flow cytometer. A single-file microsphere suspension passes by two lasers. A 635-nm laser excites the red and infrared fluorochromes impregnated in the microspheres, which allows the classification of the bead and therefore the identity of the probe-target being analyzed. A 532-nm laser excites reporter fluorochromes such as *R*-phycoerythrin and Alexa 532 to quantify any hybridization that occurs on the microsphere (Fig. 8C).

Several chemistries have been developed for nucleic acid detection by suspension bead arrays, including direct DNA hybridization, competitive DNA hybridization, and solution-based chemistries with microsphere capture (48). In direct DNA hybridization, PCR amplicons hybridize directly to probe capture sequences immobilized on the microspheres (Fig. 8B) (8, 179). Generally, a biotinylated primer is used during amplification, which allows streptavidin–*R*-phycoerythrin to bind and label hybridized microspheres. Competitive DNA hybridization utilizes unlabeled PCR amplicons and biotinylated competitor oligonucleotides. In contrast to the direct hybridization method, competitive DNA hybridization yields high fluorescence in the absence of target DNA. When target DNA is present, it binds the labeled competitor DNA, which, in turn, is not available to hybridize to the microsphere, yielding low fluorescence. Allele-specific primer extension (ASPE) or target-specific primer extension (TSPE), oligonucleotide ligation assay (OLA), and single-base-chain extension (SBCE) are solution-based chemistries coupled with subsequent microsphere capture. By exploiting the natural properties of DNA polymerases and ligases, these chemistries incorporate a capture sequence during the solution-based reaction (48). Both ASPE or TSPE and OLA use a capture primer, which contains a unique 5′ sequence followed by a target-specific sequence. In ASPE and/or TSPE, the primer can be extended by DNA polymerase only if target DNA is present to supply the complementary base for the 3′ nucleotide. The label in ASPE and/or TSPE is provided by a biotinylated deoxynucleotide triphosphate. The OLA reaction is ligase dependent. In addition to the capture primer, a biotinylated probe homologous to target DNA is present during an OLA. The capture primer and reporter probes can be ligated only if target DNA is present in the sample. Used for multiplex SNP detection, SBCE requires independent reactions for each nucleotide query. For every SNP being interrogated, one probe with a unique capture sequence is used to assay the possible alleles in separate wells containing a different dideoxynucleoside triphosphate per well (24). When the capture and target sequences are homologous, a biotinylated dideoxynucleoside triphosphate is incorporated, thereby terminating further extension.

The solution-based chemistries described above all take advantage of universal microspheres with nonspecific capture

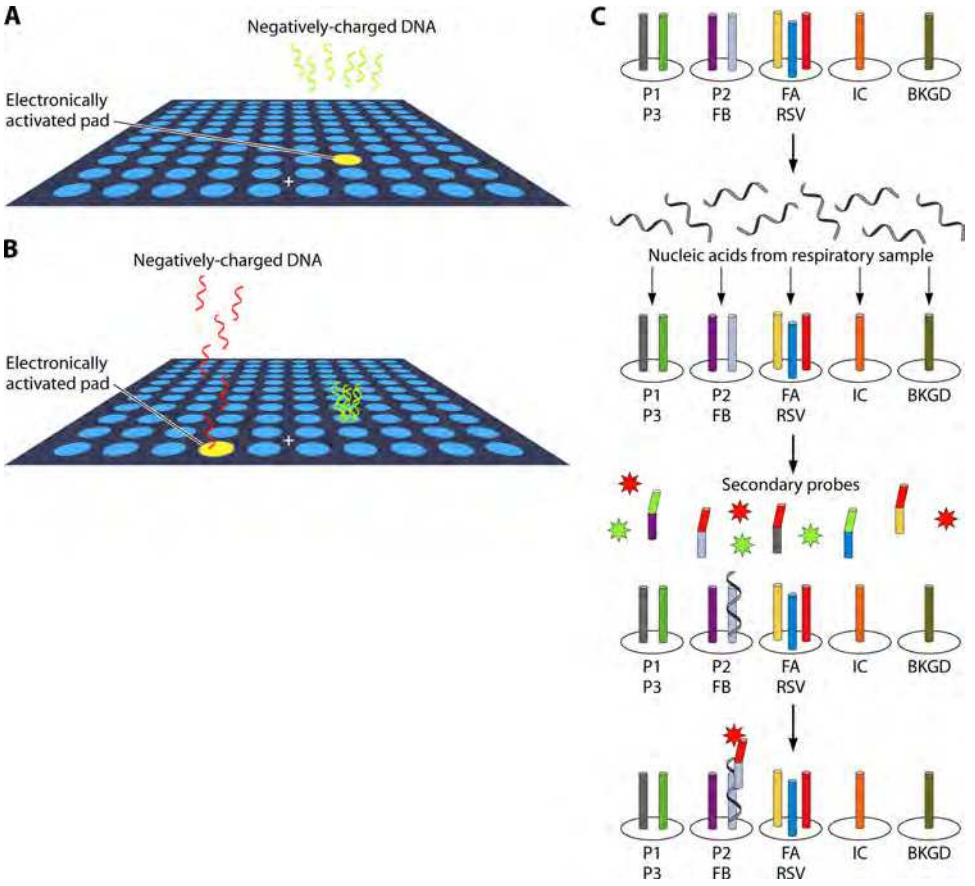

FIG. 7. Electronic microarray. (A) A positive electric current is applied to test sites, facilitating the active movement and concentration of negatively charged DNA probes to the activated locations. (B) Once the first probe is bound to its targeted location(s) by streptavidin-biotin bonds, the test site(s) can be deactivated, and current can be applied to a different test site. This process is repeated until all the probes are arrayed. (C) Nanogen's RVA ASR. Upon application of the probes to targeted test sites, extracted and amplified nucleic acids from a respiratory sample passively hybridize to the microarray surface. If hybridization occurs, secondary probes that are specific for the target and that contain a nonspecific detector sequence will bind. Secondary fluorescent detector oligonucleotides are used to measure positive hybridization reactions. Multiple probes can be used per site when multiple fluorophores are incorporated. P1, parainfluenza virus type 1; P2, parainfluenza virus type 2; P3, parainfluenza virus type 3; FB, influenza B virus; FA, influenza A virus; RSV, respiratory syncytial virus; BKGD, background. (Images courtesy of Nanogen.)

sequences. The first universal sequences used to tag microspheres were ZipCode/cZipCode capture sequences originally used with SBCE in SNP genotyping assays (24, 84, 192, 219). The 25-bp ZipCode sequences are based on random genomic sequences from *Mycobacterium tuberculosis* (24). A unique ZipCode sequence is included in the 5′ end of the capture probe used in the chemistries described above, while microspheres are tagged with the complementary sequence (cZipCode). Additional sets of universal capture sequences have been developed, including those by Tm Biosciences (xTAG; Luminex Molecular Diagnostics, Inc., Toronto, Canada) and EraGen (Madison, WI) (EraCode) (90). The sequences of the xTAG (also Tag-It and FlexMAP) system consist of 3 of the 4 nucleotides, thereby decreasing the likelihood of nonspecific hybridization to naturally occurring sequences. Since all of the xTAG sequences are thermodynamically matched, variability in hybridization efficiency is not an issue. The xTAG universal bead technology is used for all commercial assays available through Luminex. Based on the expanded genetic alphabet of MultiCode technology, EraCode sequences incorporate synthesized isoguanosine and 5-Me-isocytosine bases. EraCode sequences are highly specific since the isoguanosine and 5-Me-isocytosine bases will pair with each other but not naturally occurring bases.

Although the feature density of suspension bead arrays is the lowest of all the platforms reviewed, advantages abound that make this platform the most practical for clinical microbiology applications. The availability of universal bead sets and their inherent flexibility make the development of user-defined applications feasible and relatively inexpensive. Although users must carefully validate the positive fluorescent threshold for each analyte in the multiplex, user-defined bead-based assays provide experienced users a multitude of clinically relevant applications (see below). Importantly, in 2008, Luminex obtained FDA clearance for the first infectious-disease suspension bead array (xTAG RVP), which detects 12 respiratory viruses and subtypes (106, 132). Although analyte-specific reagents (ASRs) also exist, the availability of FDA-cleared products is a critical step in getting this technology into less-experienced diagnostic microbiology laboratories. However, many established clinical molecular microbiology laboratories rely heavily on real-time PCR, which has minimal contamination

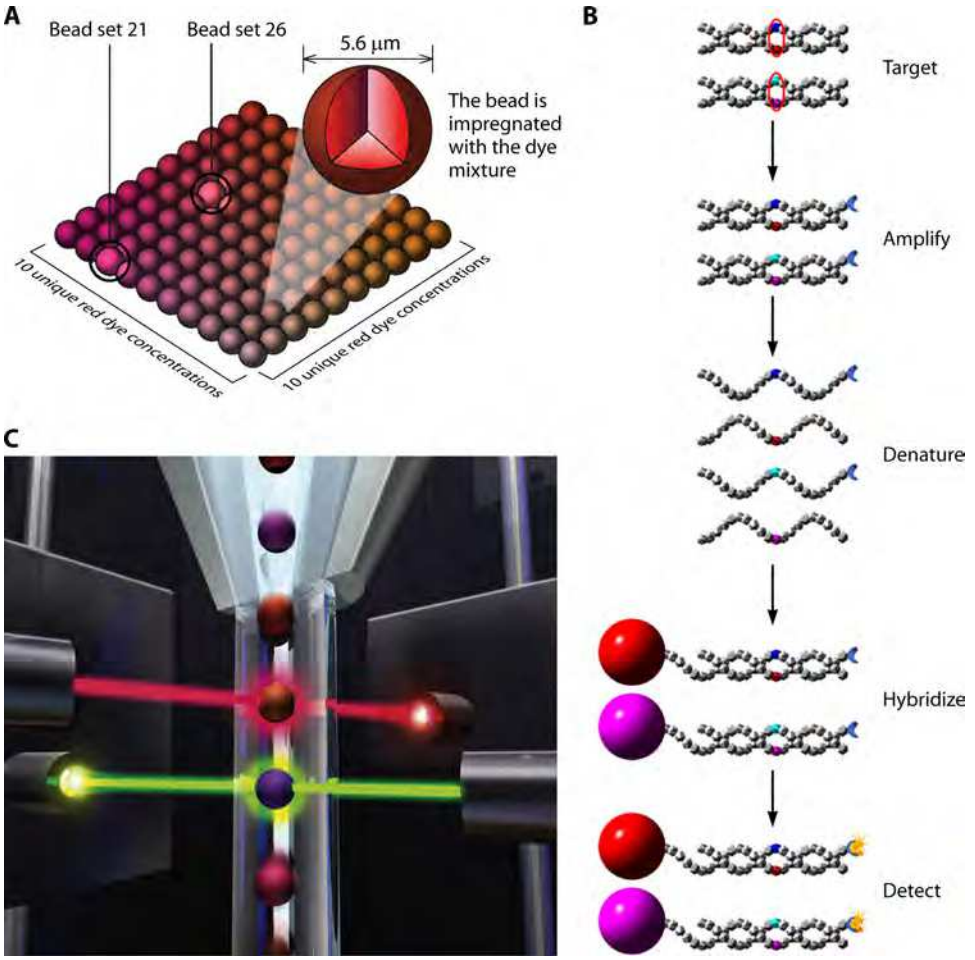

FIG. 8. Suspension bead array. (A) Microspheres 5.6 μm in diameter are filled with different relative concentrations of an infrared dye and a red dye to create 100 beads, each with a unique spectral identity. (B) Potential targets are amplified using a biotinylated primer and then denatured and hybridized to microspheres tagged with target-specific sequence probes. Probe-target hybridization is measured using a streptavidin-bound green fluorophore. (C) Flow cytometry is used to analyze the microsphere suspension. A red laser is used to determine the spectral identity of the bead and, therefore, the probe being analyzed. The reporter fluorochrome is excited by a green laser, which quantifies the probe-target reaction on the microsphere surface. (Panels A and C courtesy of Luminex Corporation; panel B adapted from reference 48 with permission from Elsevier [copyright Elsevier Inc. 2006].)

risks. In contrast, the opening of postamplification tubes and the subsequent pipetting steps in the workflow of suspension arrays increase the risk for intra- and interrun contamination. Careful consideration should be paid to contamination control measures and the reestablishment of postamplification laboratory space in the era of real-time PCR. Nonetheless, the relative simplicity, powerful multiplexing capabilities, and affordability of suspension bead arrays make this platform the most attractive for high-throughput nucleic acid detection in clinical infectious disease diagnostics.

POTENTIAL APPLICATIONS IN CLINICAL MICROBIOLOGY

Microarray technology has been used for over a decade to investigate the differential gene expression of pathogens. Although gene expression analyses have contributed significantly to our understanding of pathogenic mechanisms, pathogen responses to environmental stimuli, and host-pathogen interactions, one could argue that the data from these investigations have little direct impact on diagnostic microbiology. However, as outlined below, microarray technology has been applied to the detection and identification of various pathogens, pathogen discovery, antimicrobial resistance monitoring, and strain typing. In addition, the monitoring of host responses to infection and therapy represents a burgeoning field that, when coupled with pathogen-specific detection and monitoring, will be the ultimate diagnostic platform for infectious diseases.

Microbial Detection and Identification

Perhaps the most promising area in applying microarray technology in clinical microbiology is the use of low- or middle-density microarrays for the simultaneous assessment of large numbers of microbial genetic targets (64, 183). Specific microbial gene amplification by either a broad-range or a multiplex PCR prior to microarray analysis enhances test sensitivity. The amplification of universal microorganism targets by broad-range PCR followed by sequencing analysis has been considered a standard procedure (190); however, microarrays have

emerged as potential tools for bacterial detection and identification given their high parallelism in screening for the presence of a wide diversity of genes. The most commonly used gene targets have been the 16S bacterial and 28S fungal and intergenic transcribed spacers (ITSs) in rRNA genes, and microarray technology has been incorporated to compensate for the time-consuming sequencing identification procedure (190). An oligonucleotide microarray targeting the 16S rRNA gene was developed for the detection of a panel of 40 predominant human intestinal bacterial pathogens in human fecal samples (208). Assays using broad-range PCR incorporated with microarrays have been shown to allow rapid bacterial detection and identification with positive blood cultures (5, 128). A similar procedure was developed and used for the rapid diagnosis of bloodstream infections caused by common bacterial pathogens in the pediatric and general populations (32, 173). PCR amplification, in combination with an oligonucleotide microarray, was used to identify *Bacillus anthracis* based on the rRNA ITS region (144). Several studies reported the use of microarrays to identify pathogenic yeasts and molds by targeting the ITS regions in fungal rRNA genes (80, 81, 110). Recently, a DNA microarray was established to detect and identify 14 commonly encountered fungal pathogens in clinical specimens collected from neutropenic patients (178).

The key for broad-range PCR amplification followed by microarray identification to work is to target the right gene. It is critical to use a gene "broad" enough so that most related microorganisms can be covered in one amplification reaction. On the other hand, the targeted gene should possess enough polymorphic information to supply sufficient discriminatory power to differentiate and characterize related microorganisms. Degenerate primer sets can be designed to increase the coverage of relatively variable genes. Other universal bacterial genes have been used to detect and identify organisms using microarrays. For mycobacterial detection and identification, the *gyrB*, *rpoB*, and *katG* genes have been targeted by using microarrays (61, 197). Microarrays targeting the 23S rRNA and *gyrB* genes for bacterial detection and identification using clinical specimens have been described (92, 102, 136). In addition to bacterial and mycobacterial organisms, microarrays following broad-range PCR amplification have been used to detect and identify fungal, parasitic, and viral pathogens (43, 101, 210).

Microarrays have also been incorporated with multiplex PCR amplification for the simultaneous detection and identification of a panel of microbial pathogens in a single reaction. Khodakov et al. described a novel microarray-based approach for the simultaneous identification and quantification of human immunodeficiency virus type 1 (HIV-1) and hepatitis B and C viruses in donor plasma specimens (96). A microarray technique for the detection and identification of enteropathogenic bacteria at the species and subspecies levels was developed, covering pathogenic *Escherichia coli*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Salmonella enterica*, *Campylobacter jejuni*, *Shigella* spp., *Yersinia enterocolitica*, and *Listeria monocytogenes* (220). A microarray-based multiplexed assay was developed to detect foot-and-mouth disease virus with rule-out assays for two other foreign animal diseases and four domestic animal diseases that cause vesicular or ulcerative lesions that are indistinguishable from those of foot-and-mouth disease virus infection of cattle, sheep, and swine (111). Bøving et al. reported the development of a novel multiplex PCR with product detection by the Luminex suspension array system covering a panel of bacterial and viral pathogens causing meningitis. This system detected and identified nine microorganisms including *Neisseria meningitidis*, *Streptococcus pneumoniae*, *E. coli*, *Staphylococcus aureus*, *L. monocytogenes*, *Streptococcus agalactiae*, herpes simplex virus types 1 and 2, and varicella zoster virus directly from cerebrospinal fluid (15). The ResPlex I system, manufactured by Qiagen (Valencia, CA), was used to detect a panel of bacterial pathogens related to community-acquired pneumonia from tracheal aspirates collected from hospitalized antibiotic-treated children. The data indicated that the ResPlex I system significantly enhanced the pathogen-specific diagnosis of community-acquired pneumonia in children (39). This gene-specific, multiplex amplification followed by a microarray identification system provides a great example for additional clinical diagnostic applications such as the detection and differentiation of respiratory viral pathogens, which is described in detail below.

Respiratory Viral Pathogen Detection in Connection with Multiplex PCR Amplification

Respiratory infections caused by a panel of bacterial, viral, and fungal pathogens usually present with similar signs and symptoms that are nearly indistinguishable by clinical diagnosis. Simultaneous testing for all possible pathogens is an efficient means to obtain a conclusive result. In addition, assaying for all potential pathogens may yield information regarding possible coinfections or induced secondary infections. The first promising respiratory microarray system was described in 2002, which incorporated 1,600 unique 70-mer-long oligonucleotide probes covering approximately 140 viral genome sequences (206, 207). This ViroChip system was used to identify the severe acute respiratory syndrome virus as a coronavirus (163), for the discovery of a human parainfluenza virus type 4 infection associated with respiratory failure (27) and human coronavirus and rhinovirus in nonasthmatic patients (98), and for the diagnosis of a human metapneumovirus causing critical respiratory illness (26). A resequencing microarray based on the Affymetrix GeneChip platform that used short oligonucleotides to simultaneously provide both species-level and strain-level identification of respiratory pathogens was developed (127, 209). The system was able to detect and identify 26 respiratory pathogens including the novel influenza virus subtypes H5N1 and H1N1 (115, 116). Another comprehensive and panmicrobial microarray, the GreeneChipResp system, was developed and later used for the detection of respiratory viruses and the subtype identification of influenza A viruses (150, 157). In addition to the detection and identification of respiratory pathogens, several formats of microarrays that detect the whole coronavirus genus (42) and detect and type influenza viruses (113, 131, 170, 227) have been described.

Several commercial products are available for the detection of a panel of respiratory viruses, which incorporate microarrays as the identification method (18, 106, 109, 112, 126, 132, 143, 148, 160, 185). These products include the Infiniti RVP from AutoGenomics, Inc. (Carlsbad, CA); the MultiCode-PLx RVP from EraGen Biosciences (Madison, WI); the ResPlex II assay

TABLE 2. Comparison of commercially available, microarray-based kits for detection and identification of respiratory viruses[a]

Product	Company	Viruses and/or genotypes detected	Amplification platform(s)	Microarray platform	Characteristic(s)	Reference(s)
Infiniti RVP	AutoGenomics, Inc. (Carlsbad, CA)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV-A, RSV-B, hMPV-A, hMPV-B, RhV-A, RhV-B, EnV, CoV, and Adv	Multiplex PCR and RT-PCR	Infiniti analyzer (solid chip)	The detection step by the Infiniti analyzer is completely automatic	160
MultiCode-PLx RVP	EraGen Biosciences (Madison, WI)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV, hMPV, RhV, AdV, and CoV	Multiplex PCR and RT-PCR	Luminex (liquid chip)	Universal beads used in detection employ EraCode sequences	109, 143
ResPlex II assay	Qiagen (Valencia, CA)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV-A, RSV-B, hMPV, RhV, EnV, and severe acute respiratory CoV	Multiplex RT-PCR (Tem-PCR)	Luminex (liquid chip)	A unique Tem-PCR allows large numbers of targets included in one reaction without significant loss of sensitivity	18, 112
NGEN respiratory virus ASR	Nanogen (San Diego, CA)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, and RSV	Multiplex RT-PCR	NanoChip (solid chip)	Probe labeling, target capture, and detection are accomplished using electronic microarray technology	112, 185
xTAG RVP	Luminex Molecular Diagnostics (Toronto, Ontario, Canada)	Flu-A, Flu-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV-A, RSV-B, hMPV, AdV, EnV, CoV, and RhV	Multiplex PCR and RT-PCR	Luminex (liquid chip)	TSPE is used in combination with universal detection beads	126, 132, 148

[a] Abbreviations: Tem, target-enriched multiplex; Flu, influenza virus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; RhV, rhinoviruses; EnV, enteroviruses; CoV, coronavirus; RT, reverse transcription.

from Qiagen (Valencia, CA); the Ngen respiratory virus ASR assay from Nanogen (San Diego, CA); and the xTAG RVP from Luminex Molecular Diagnostics (Toronto, Canada). Table 2 contrasts these commercially available kits. Among them, the EraGen, Qiagen, and Luminex molecular diagnostics systems incorporate multianalyte profiling by a liquid-bead microarray system developed by Luminex (discussed above) (48). Specific applications of this technology for nucleic acid detection include SNP genotyping, genetic disease screening, gene expression profiling, and microbial detection and typing. Although suspension bead arrays are amenable to high-throughput nucleic acid detection, the efficiency of the front-end multiplex PCR amplification limits the number of pathogens that can be included in one reaction. With the implementation of novel multiplex amplification procedures, numbers of targets included in one reaction can be significantly increased without a significant loss of sensitivity (112, 166).

Simultaneous Detection and Typing of Human Papillomaviruses

Persistent infection with known high-risk human papillomavirus (HPV) types is a significant risk factor for cervical cancer and is increasingly being recognized as playing a role in other cancers. Recently, HPV vaccines have demonstrated effectiveness in preventing type-specific persistent infection and disease. To monitor the impact of vaccine implementation strategies, determine type-specific persistence, and evaluate the clinical significance of coinfection with multiple genotypes, HPV testing will require type-specific results. A high-throughput, sensitive, specific, and reproducible HPV detection and typing assay is therefore highly desirable. Most established HPV typing assays are based on consensus PCR to amplify the relatively conserved L1 gene region with hybridization, restriction enzyme digestion, or sequencing of the amplicon to determine type(s). Recently, several studies were aimed at evaluating the usefulness of microarray technology for the simultaneous detection and typing of HPV in routine clinical specimens. A user-developed HPV DNA microarray for high-risk HPV genotyping was evaluated by using a panel of malignant and nonmalignant cervical smears. This approach provides the potential to improve the clinical management of patients with cervical cytological abnormalities (3). Several systems that combine multiplex PCR amplification and microarray identification have been reported to provide rapid and reliable diagnostic tools for HPV detection and typing that are amenable to automation (73, 119, 140, 145). Additional studies that incorporated microarrays to detect and characterize high-risk mucosal HPV types (66), betapapillomavirus types (65), and the frequencies of 23 HPV types in women with and without cytological anomalies (193) have been reported. A

novel DNA detection assay incorporating the Luminex suspension array was reported and was applied to the genotyping of HPV in cervical samples (141). The molecular inversion probe microarray assay, originally applied to large-scale human SNP detection, has been used for HPV detection and typing to demonstrate the potential of the method for the detection and characterization of any microbe (1).

Rapid Detection and Characterization of Methicillin-Resistant *Staphylococcus aureus*

Staphylococcus aureus, including methicillin-resistant *S. aureus* (MRSA), is an important pathogen in hospitals and, increasingly, in communities around the world. Advanced laboratory techniques, including diagnostic microarray analysis, have been sought to rapidly identify staphylococcal isolates and determine antimicrobial susceptibility patterns. DNA microarray analyses of large samples of clinically characterized community-acquired MRSA strains have been reported, which provide broad insights into evolution, pathogenesis, and disease emergence (57, 99, 168). DNA microarrays based on the Array-Tube platform (ClonDiag Chip Technologies, Jena, Germany) have been used for characterizing and genotyping staphylococcal DNA, including their relevant resistance determinants and virulence factors (137–139). Microarrays provide a valuable epidemiological tool for the detailed characterization of MRSA isolates and comparison of strains at a global level (137). In addition, several techniques incorporating peptide and/or nucleic acid probes and conventional and real-time PCR have been used to take advantage of the rapid enrichment of automated blood culture instruments to rapidly identify MRSA from flagged blood cultures when gram-positive cocci in clusters are observed. The combination of novel multiplex PCR amplification and suspension bead array detection (StaphPlex) for the rapid detection and characterization of staphylococci directly from positive blood culture bottles was described (191). The StaphPlex system provides simultaneous staphylococcal identification, antibiotic resistance determinant detection, detection of Panton-Valentine leukocidin, and determination of staphylococcal cassette chromosome *mec* types I to IV within 5 h. This approach potentially impacts antibiotic usage when gram-positive cocci in clusters are detected by reducing the unnecessary use of vancomycin, which is often used empirically to treat patients until susceptibility results are available (191). A similar system (MVPlex) was developed and was used to screen for MRSA in nasal swabs (154). Notably, the MVPlex system detects 13 different molecular targets including vancomycin-resistant *Enterococcus*.

Determination of Antimicrobial Drug Resistance

Another successful application of microarray techniques in clinical microbiology is the determination of antimicrobial resistance by simultaneously detecting a panel of drug resistance-related mutations in microbial genomes (21, 36, 72, 153, 196, 224, 225). The emergence of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis and time-consuming phenotypic antimycobacterial susceptibility procedures have stimulated the pursuit of microarray platforms in antituberculosis drug resistance determinations. High-density DNA oligonucleotide arrays have been used for parallel species identification and rifampin resistance-related mutations in mycobacteria (197) and, more specifically, for the detection of *M. tuberculosis* strains that are resistant to rifampin (40, 176, 222) or isoniazid, kanamycin, streptomycin, pyrazinamide, and ethambutol (41, 189, 204). Oligonucleotide microarrays were developed to analyze and identify drug-resistant *M. tuberculosis* strains, and it was found that the results were comparable with those of standard antimicrobial susceptibility testing (69, 134, 184). A low-cost and -density DNA microarray was designed to detect mutations that confer isoniazid and rifampin resistance in *M. tuberculosis* isolates. The low-cost and -density array protocol takes 45 min after PCR amplification, with only minimal laboratory equipment required (7). Antonova and colleagues developed a method for the detection and identification of mutations in the *M. tuberculosis* genome determining resistance to fluoroquinolones by hybridization on biological microchips (6). A recently developed QIAplex system combines a novel multiplex PCR amplification and suspension bead array identification for the simultaneous detection of 24 *M. tuberculosis* gene mutations responsible for resistance to isoniazid, rifampin, streptomycin, and ethambutol (63). Several studies that detected antibiotic resistance-related mutations in bacterial genomes have been reported (2, 68, 153, 202, 221, 225).

Microarray-based techniques face several application challenges to determine antimicrobial resistance in the clinical setting. First, genomes of some pathogens continue to mutate under natural and therapeutic selective pressures, which is well demonstrated by HIV-1. An Affymetrix microarray was developed to provide HIV-1 antiretroviral-drug-resistant profiles (104, 198, 213). The product was discontinued due to rapidly emerging HIV-1 genome mutations. The company now has a comprehensive, high-density microarray available to identify every mutation in resistance-related HIV-1 genomes. Second, molecular mechanisms for many antimicrobial drug resistances remain to be discovered while novel resistance genes and mutations continue to emerge. It takes considerable time and effort to decipher all of the resistance-related mutations and transfer the basic science findings to clinical applications. For *M. tuberculosis*, until such knowledge is available, the currently used phenotypic methods for identifying resistance will continue to play an invaluable role in optimizing the therapy of persons with tuberculosis.

Microbial Typing

Numerous studies that use microarrays for microorganism typing by taking advantage of its simultaneous detection of a variety of genomes have been reported. The accurate identification and prompt typing of pathogens causing diarrheal diseases are critical for directing clinical intervention, including appropriate antibiotic administration, and facilitating epidemiological investigations. Microarray-based approaches along with other genetic approaches that can be used to support or replace the classical serotyping method for several conventional diarrhea bacterial pathogens have already been offered. The use of microarrays has included *Salmonella*, *Helicobacter*, and *Campylobacter* species (46, 56, 155, 164, 201, 212). PCR followed by a microarray hybridization step has been used for

the detection and typing of *E. coli* virulence genes (28, 199). A serotype-specific DNA microarray for the identification of clinically encountered *Shigella* and pathogenic *E. coli* strains was recently described (114). Diagnostic microarrays based on the ArrayTube format were devised for virulence determinant detection as well as for protein-based serotyping of *E. coli* (4, 100). A novel ArrayTube assay, which incorporates oligonucleotide DNA probes representing 24 of the most epidemiologically relevant O antigens and 47 H antigens, has been described for fast DNA serotyping of *E. coli* (9). Microarrays have also been used to characterize and type other gastroenteritis-causing viral pathogens including rotavirus, norovirus, and astrovirus (29, 77, 86, 103, 123). Beyond diarrheal illnesses, Pas et al. reported the comparison of reverse hybridization, microarray, and sequence analysis for hepatitis B virus (HBV) genotyping, suggesting that the InnoLipa HBV genotyping strip assay, a microarray-based system, detected dual infections and was an easy and quick tool for HBV genotyping (152).

Microbial Gene Expression Profiling

The quantification of multiple microorganisms simultaneously using microarray techniques has rarely been reported, probably due to technical difficulties. Instead, the detection and monitoring of the gene expressions of individual microorganism genomes during infection have begun to generate meaningful data (16, 62). Whole-genome microarrays for *M. tuberculosis* were first described using the amplicon arrays developed at Stanford to define gene expression responses to isoniazid and ethambutol (214). Subsequently, microarrays have been used to monitor *M. tuberculosis* gene expression responses to a variety of environmental conditions and exposure to antibiotics (11, 93, 161, 180, 203). *M. tuberculosis* gene expression patterns associated with resistance and susceptibility and mycobacterial survival during infection have been investigated by use of oligonucleotide microarrays (60, 94, 165). The transcriptional profile of *M. tuberculosis* from human lung samples has been studied; during pulmonary tuberculosis, *M. tuberculosis* actively transcribes a number of genes involved in active fortification and evasion from host defense systems (158). Microbial candidate genes have been studied by differential-expression microarrays for discrimination between infection and disease caused by *M. tuberculosis* (87).

Gene expression profiles of other bacterial and fungal infections have also been studied by microarrays (17, 37, 44, 45, 67, 85, 121, 124, 162, 181, 218). A concordance of the gene expression data between intracellular *Shigella* and *Salmonella* has been noted, although they colonize different niches inside the cell (124). So far, most studies of microbial gene expression profiling have been limited in research. Techniques used in these studies need to be validated to ensure that sufficient amounts of mRNA are extracted such that gene expression data are not compromised. The host-pathogen interactions that define a disease are clearly complex, and other genotypic and phenotypic data need to be integrated to clarify the intricate cross talk from host to pathogen and the environmental cues that lead to the expression of bacterial virulence factors in vivo. Nevertheless, microbial gene expression profiles reveal a complete picture of the metabolic state of bacteria under a particular condition, thereby providing a potential tool for the diagnosis and monitoring of microbial infection and disease.

Host Gene Expression Profiling during Microbial Infections

Pathogen-induced phenotypic changes in a host are often accompanied by marked changes in host gene expression. Genome-wide expression profiling of the hosts, in addition to the pathogens, has become increasingly important for studying host-pathogen interactions (88). The advent of microarray technology has greatly expanded our ability to monitor changes in host gene expression. The cellular transcriptional response to human cytomegalovirus was globally monitored with an oligonucleotide array in 1998 (223). Subsequently, oligonucleotide microarrays have been generated to measure host gene expression profiles in response to *E. coli*, *Candida albicans*, *L. monocytogenes*, influenza virus, and respiratory syncytial virus infections (34, 58, 82). A microarray was generated to incorporate a series of host response genes including those involved in inflammation and chemotaxis as well as those involved in the synthesis of prostaglandins, Toll-like receptors, and T-cell regulation (97). Such a microarray system has been used to determine immune responses in normal human monocytes after fungal pathogen infections and antifungal drug inoculation (35, 97, 174). Microarrays have been implemented to generate gene expression profiles for viral hepatitis infections, which provide enormous diagnostic and therapeutic potential (75, 76, 147, 171, 211).

Several studies that used microarray-based techniques to detect and characterize host gene expression profiles for sepsis have been reported. Microarray technology was first used to analyze tissue-specific changes in gene expression induced by sepsis in animal models (33). Subsequently, numerous studies that used host gene expression profiling toward sepsis diagnostics, pathogen type differentiation, and clinical outcome prediction have been described (Table 3) (55). The rapid determination of a host sepsis transcriptome provides an early differential diagnosis and clinical outcome prediction. Current microarray-based techniques using host gene expression profiles are limited due to the background variation among and within the individuals studied and poor quality control built into the microarrays. Therefore, the gene expression differences notified by the microarrays have to be verified by gene-specific quantitative real-time PCR assays (95).

Host Genomic Polymorphism Determination

When infections, especially chronic infections, are viewed as horizontally acquired genetic diseases, it makes sense to view the pathogen and host as an integrated system. Host genetic polymorphisms that influence the host immune response to infectious agents, thereby determining susceptibility to certain diseases and pathological conditions, which has been well explored in sepsis, have been described (118). SNP analysis is a powerful tool for the mapping and diagnosing disease-related alleles. While sequencing remains the "gold standard" to determine host genetic variabilities, microarray-based techniques may become a simple, rapid, automatic, and user-friendly format for screening and detecting a large panel of related SNPs simultaneously. An Affymetrix HuSNP assay was used to study the role of human

TABLE 3. Selected sepsis studies using microarray-based host gene expression

Chip used	Subject(s)	Main findings and conclusions	Reference
Atlas array, Clontech Laboratories (Mountain View, CA)	Mouse	Microarray technology provides a powerful new tool for rapidly analyzing tissue-specific changes in gene expression induced by sepsis	33
Hu95aVer2 GeneChip, Affymetrix (Santa Clara, CA)	Adult patients	The host inflammatory responses to gram-negative and gram-positive stimuli share some common response elements but also exhibit distinct patterns of cytokine appearance and leukocyte gene expression	55
Image consortium libraries, Livermore National Laboratory (Livermore, CA)[a]	Mouse	Both gram-positive sepsis and gram-negative sepsis share a final common pathway involved in the pathogenesis of sepsis, but certain genes are differentially expressed under distinct regulation	221
Arraytor human 500-1 cDNA, SIRS-Laboratory (Jena, Germany)	Adult patients	Microarrays can identify typical gene expression profiles for blood samples from patients with severe sepsis	156
Hu 133A and 133B GeneChip, Affymetrix	Healthy adult blood leukocytes receiving bacterial endotoxin stimulus	Human blood leukocyte response to acute systemic inflammation includes the transient dysregulation of leukocyte bioenergetics and modulation of translational machinery; these findings provide insight into the regulation of global leukocyte activities as they relate to innate immune system tolerance and increased susceptibility to infection in humans	22
MGU74Av2 GeneChip, Affymetrix	Mouse	A(2A)R blockade may be useful for treatment of infection and sepsis	142
HG-U133A GeneChip, Affymetrix	Adult patients	Blood transcriptional profiling is a valuable approach not only for patient stratification but also to identity new genes possibly involved in sepsis pathophysiology	149
Mouse 430 2.0 GeneChip, Affymetrix	Mouse	T-cell receptor signaling and mitogen-activated protein kinase signaling were significantly altered by sepsis	129
U74Av2 GeneChip, Affymetrix	Mouse	Sepsis induces common inflammatory response gene changes in mouse leukocyte gene expression that can be used to diagnose sepsis	31
Adelaide Microarray, Compugen, San Jose, CA)	Adult patients	The signature genes reflect suppression of neutrophils' immune and inflammatory function by sepsis; gene expression profiling therefore provides a novel approach to advance our understanding of the host response to sepsis	188
430A GeneChip, Affymetrix	Mouse	Sepsis induces alterations in balance of pro- and antiapoptotic transcriptional networks, and bcl-2 overexpression improves survival in sepsis	205
U133 Plus 2.0 GeneChip, Affymetrix	Pediatric patients	Genome-level alterations of zinc homeostasis may be prevalent in clinical pediatric septic shock	216
U133 Plus 2.0 GeneChip, Affymetrix	Adult patients	Sepsis has a unique gene expression profile that is different from that for uninfected inflammation and becomes apparent prior to expression of the clinical sepsis phenotype	89
U133 Plus 2.0 GeneChip, Affymetrix	Adult patients	Toll-like receptors and downstream signaling genes are differentially expressed in critically ill patients developing sepsis compared with those with sterile inflammation; these expression differences occur before phenotype-based diagnosis of clinical sepsis	120
U133 Plus 2.0 GeneChip, Affymetrix	Adult patients	There was evidence of sepsis-related immunosuppression and reduced inflammatory response in mononuclear cells on a transcriptome level; these characteristic transcriptional changes can be used to aid the diagnosis of sepsis	187

[a] Distributed by ResGen Invitrogen (Huntsville, AL).

genomic SNPs in the pathogenesis of human parvovirus B19 infection, and relevant SNPs revealed by the microarray study were further confirmed by allele-specific real-time PCR assays (95). A microarray was developed for the simultaneous genotyping of four host SNPs associated with the therapeutic effect of interferon in hepatitis C virus patients (186). These preliminary data suggest that a genetic predisposition is associated with the pathogenesis and development of microbial infections.

CONCLUDING REMARKS

Microarrays have the unprecedented potential to simultaneously detect and identify thousands of microbial genes, which provides another evolutionary technical advance in the field of clinical microbiology. Although, historically, microarrays have been used largely for gene expression studies, microarrays have gradually been applied in the detection and

characterization of microbial pathogens, determination of antimicrobial resistance, typing of microbial pathogens, and monitoring of microbial infections by investigating host genomic expression and polymorphism profiles. Even with these major advances, the potential power behind microarray applications in clinical microbiology has yet to be fully realized. The ability to detect multiple pathogens and/or monitor the variability of normal microbial populations in a disease process could transform our current understanding of infectious diseases. In addition, massively parallel sequencing performed by microarray analysis offers the opportunity of sequencing directly from complex clinical specimens. This metagenomics approach will allow a comprehensive analysis of every nucleic acid in the specimen. For these robust applications, high-density microarray platforms must be able to transition from translational research laboratories to the clinical laboratory. It is unlikely that traditional, planar microarrays will soon appear in clinical microbiology laboratories due to their high cost, relative lack of flexibility, and limited throughput. The ideal microarray platform for the diagnostic laboratory is a low- to medium-density array that offers limited, reliable, and straightforward results without the need for sophisticated equipment and data management (133). Indeed, platforms that have begun to meet these criteria have been developed, such as electronic microarrays and suspension bead arrays.

With the potential power of microarray analysis comes abundant challenges, particularly in relation to the diagnostic laboratory. Several critical issues need to be resolved before microarray-based techniques can be widely implemented in clinical microbiology services. Due to the potential variability in multiple steps included in the microarray analysis, it is difficult to compare quantitative data between, and even within, microarray experiments. Substantial obstacles still exist along the entire spectrum of preanalytical-to-postanalytical analysis. Heterogeneous clinical specimens present unique challenges with respect to sensitivity, specificity, quantification, and data analysis of microarrays that are not encountered during the analysis of pure cultures. In addition, optimization of extraction, labeling, and hybridization; incorporation of appropriate quality controls, design, and implementation of clinical validation studies; and management and interpretation of data remain challenges in a clinical setting. Moreover, laboratories must account for microarray reproducibility in production and analysis, cost of implementation, acquisition of appropriately skilled laboratorians, as well as intellectual property and reimbursement issues. Compared to real-time PCR, microarray analysis requires additional manipulations including hybridization and washing, which increase the contamination risk and the amount of hands-on time needed, both steps backwards in diagnostic molecular microbiology.

Although improvements are still needed to make the majority of microarray applications amenable to clinical microbiology laboratories, the future role of these robust technologies in diagnostic microbiology is indisputable. Microarray-based analyses will revolutionize infectious disease diagnostics through the detection and identification of previously unknown or unsuspected pathogens, by transforming our current view of multiplexed laboratory testing, and by expanding pathogen detection to include bacterial population-based analyses and host-specific responses (135). As more pathogen genomes and targeted genes are sequenced, costs associated with microarray production decrease, and FDA-cleared products become available, diagnostic applications of microarray-based analyses will continue to expand. As PCR has done in the last 25 years, and more recently real-time PCR, microarray technology will undoubtedly transform the diagnostic capabilities of clinical laboratories, ushering us into a new molecular revolution.

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Melissa B. Miller, Ph.D., D(ABMM), is an Assistant Professor of Pathology and Laboratory Medicine at the University of North Carolina at Chapel Hill School of Medicine. She is also the Director of the Molecular Microbiology Laboratory and Associate Director of the Microbiology-Immunology Laboratory at the UNC Hospitals. Dr. Miller received her Ph.D. in Molecular Biology from Princeton University and completed the Medical and Public Health Microbiology Fellowship at the UNC Hospitals. She is a member of the Board of American College of Microbiology and the editorial board for the *Journal of Clinical Microbiology*. Dr. Miller was honored as the 2009 recipient of ASM's Siemens Healthcare Diagnostics Young Investigator Award. Dr. Miller's research focus is the development and assessment of molecular diagnostic assays for the detection of pathogens and the study of microbial epidemiology and antimicrobial resistance.

Yi-Wei Tang, M.D., Ph.D., is an Associate Professor of Pathology and Medicine and Director of the Molecular Infectious Disease Laboratory at Vanderbilt University Medical Center. Dr. Tang received his medical degree from Fudan University School of Medicine in Shanghai and his Ph.D. in microbiology and immunology from Vanderbilt University. He has received postdoctoral training at the U.S. Centers for Disease Control and Prevention and a clinical microbiology fellowship at the Mayo Clinic. Dr. Tang is an editor for the *Journal of Clinical Microbiology* and a fellow of the American Academy for Microbiology and of the Infectious Disease Society of America. Dr. Tang has research interests in the development and validation of molecular techniques and has published over 100 peer-reviewed original articles, reviews, and book chapters in the field of diagnostic molecular microbiology.

Exhibit 414

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DNA microarray analysis of predominant human intestinal bacteria in fecal samples

Rong-Fu Wang[a,*], Marjorie L. Beggs[b], Bruce D. Erickson[a], Carl E. Cerniglia[a]

[a]*Microbiology Division, National Center for Toxicological Research, US-FDA, 3900 NCTR Rd, Jefferson, AR 72079, USA*
[b]*Department of Geriatrics, Microarray Core Facility, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA*

Abstract

A microarray method was developed for the detection of 40 bacterial species reported in the literature to be predominant in the human gastrointestinal tract. The 40 species include seven species each of *Bacteroides* and *Clostridium*, six species of *Ruminococcus*, five species of *Bifidobacterium*, four species of *Eubacterium*, two species each of *Fusobacterium*, *Lactobacillus* and *Enterococcus*, and single species each of *Collinsella*, *Eggerthella*, *Escherichia*, *Faecalibacterium* and *Finegoldia*. Three 40-mer oligos specific for each bacterial species were designed based on comparison of the 16S rDNA sequences available in the GenBank database, and were used to make the DNA-array on epoxy slides. Using two universal primers, the 16S rRNA gene from bacteria present in fecal samples were amplified and labeled with Cyanine5-dCTP by PCR, and then hybridized to the DNA-array. After resolving some difficulties caused by sequence conflicts in GenBank and inaccurate reference strains, all 40 bacterial reference species gave positive results. The microarray method was used to screen fecal samples obtained from 11 healthy human volunteers for the presence of these intestinal bacteria. The results indicated that 25–37 of the 40 species could be detected in each fecal sample and that 33 of the species were found in a majority of the samples.
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Keywords: Microarray; Human intestinal bacteria; Fecal samples; PCR; 16S rRNA gene amplification

1. Introduction

The human intestinal microbiota is a balanced ecosystem that is important in maintaining an individual's health. It is well established that the microbiota in the human gastrointestinal tract form an extremely complex ecological community populated with over 10^{11} bacterial cells per gram of content and containing more than 400 bacterial species [1–3]. Approximately 90% of these species are obligate anaerobes in 30 different genera, and the predominant genera, as characterized by culture methods, are *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Clostridium*, *Fusobacterium*, *Ruminococcus*, *Enterococcus*, and *Peptostreptococcus* [1–6]. As the more prevalent species within these genera have been studied more closely, several have been reclassified [7–11]. Although there may be large individual variation in the proportions of the major species from person to person, depending upon diet or overall health of the host, the population sizes of different species from the same individual are relatively stable [3,6,12]. The bacteria interact with each other and with their host, both locally due to intimate contact with the intestinal mucosa, and systemically by influencing diverse responses in immunological, physiological, anatomical, metabolic, nutritional, antibiotic-resistance-gene transfer, and toxicological functions [13–16]. Intestinal microbiota are an essential component of human physiology because they act as a barrier against colonization of the gastrointestinal tract by pathogenic bacteria [17] as well as playing important roles in the digestion of dietary components and the metabolism of drugs, xenobiotics, and nutrients. They also provide compounds such as short chain fatty acids, vitamins and other essential nutrients that are later absorbed into the system [18,19].

Traditionally, the population of anaerobic bacteria in the human gastrointestinal tract was characterized by microscopic, biochemical, physiological, and selective culture plating methods of fecal samples from human subjects [5,6]. In recent years, a variety of molecular techniques have also been used to analyze the bacterial community in human fecal samples [20–29]. Molecular analysis can detect perturbations in the human intestinal microbiota in a rapid and precise manner [30,31]. Previously, we developed polymerase chain reaction (PCR) methods for the detection and quantification of predominant anaerobic bacteria in

* Corresponding author. Tel.: +1-870-543-7084; fax: +1-870-543-7307.
E-mail address: rwang@nctr.fda.gov (R.F. Wang).

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human and animal feces [32–34]. Although these techniques provided useful data, one of the limitations of our methodology was that we had to test for each bacterial species separately. Microarray technology is a powerful tool that can be used for detection of thousands of genes or target DNA sequences on one glass slide [35,36]. Most reports using microarray methods involve studies on gene expression. However, microarray methods can be adapted for the detection of bacteria and for DNA-based typing of specific pathogenic bacterial strains [35,37–40].

In this study, we described a fast and sensitive microarray method, which is able to detect 40 bacterial species that cover the predominant human intestinal bacteria typically reported in fecal samples. We used this method for the detection and identification of the predominant bacterial species in 11 healthy volunteers.

2. Materials and methods

2.1. Source of bacterial strains and culture conditions

The 40 reference strains of predominant human intestinal bacterial species used in this study were obtained from the American Type Culture Collection (ATCC) (Table 1). Anaerobic bacteria were cultured at 35 °C either in pre-reduced anaerobically sterilized (PRAS) brain heart infusion broth supplemented with vitamin K and hemin (BHI; Remel, Lenexa, KS), or on PRAS Brucella blood agar plates supplemented with vitamin K and hemin (Remel). Broth cultures were inoculated under an oxygen-free cannula using 85% nitrogen, 10% hydrogen and 5% carbon dioxide, while plate cultures were inoculated in an anaerobic gas chamber (Coy Laboratory Products Inc., Ann Arbor, MI) under 85% N_2, 10% H_2, and 5% CO_2.

2.2. Identification of bacterial species from fecal samples by culture methods

A fresh fecal sample was collected, transferred to the anaerobic chamber, and homogenized. A 1 g aliquot was suspended in saline and a 10-fold dilution series was prepared. Aliquots (0.1 ml each) were spread onto both selective and non-selective media. The media used were Brucella blood agar (BRU), Bacteroides bile esculin agar (BBE), Phenylethyl alcohol agar (PEA), Kanamycin–vancomycin–laked blood agar (LKV), Fusobacterium selective agar (FUS), Egg yolk agar (EYA), MacConkey agar, and Bile esculin azide agar (BEA). The BRU, FUS, and EYA plates (Anaerobe Systems, Morgan Hill, CA) were obtained in pre-reduced form, while the BBE, PEA, LKV, and BEA (Remel, Lenexa, KS) and MacConkey (bioMerieux, Lombard, IL) media were reduced overnight in the anaerobe chamber before use. The MacConkey and BEA plates were incubated aerobically at 35 °C, while the other plates were incubated anaerobically at 35 °C. After growth for 24–48 h, 55 colonies were picked and streaked for purification on pre-reduced tryptic soy agar plates with 5% sheep's blood (bioMerieux, Lombard, IL). Aerobic cultures were characterized by Gram-staining and analysis using the VITEK System (bioMerieux, Lombard, IL). Anaerobic cultures were characterized by Gram-staining, inoculation into the appropriate liquid medium (PY glucose or PY glucose + Tween) (Remel, Lenexa, KS), and analysis using the Microbial Identification System (MIDI Inc., Newark, DE).

2.2. Design of oligonucleotide probes and preparation of oligonucleotide-microarray slides

Three 40-mer oligonucleotides specific for each bacterial species (total 120 probes) were designed, based on comparison of the 16S rRNA gene sequences in the GenBank data library. The first consideration was to choose the regions specific to each species and the second was to consider the potential secondary structures of the oligonucleotides. The 40 target bacterial species' name with the species' numbers are listed in Table 1, but several species have been reclassified as indicated in the legend. The corresponding 120 oligonucleotide probe numbers and the sequences are also listed in Table 1. High Purity Salt Free (HPSF®) oligos were ordered from MWG-biotech (High Point, NC, USA) and used for microarray production without additional modification or purification. The oligos were suspended in buffer A (supplied by MWG-biotech) at a final concentration of 50 μM. Using an Omnigrid Printer from GeneMachines (San Carlos, CA) with one pin from Telechem International Inc. (Sunnyvale, CA), oligos were printed on epoxy slides (MWG-biotech). Post-processing for epoxy slides was according to the MWG-biotech protocol. Briefly, slides were incubated overnight at 42 °C in 40% humidity, washed for 2 min in 0.2% SDS and washed 2 × with ddH_20 at room temperature, then incubated at 50 °C for 20 min in ddH_20, washed 1 × with ddH_20 and spun dry.

2.3. PCR amplification of cyanine5 (CY5)-labeled 16S rDNA

Twenty five μl of PCR mixture was made by combining 15.6 μl of water; 2.5 μl of 10 × BSA-buffer (1 ml 10 × buffer is composed of 0.5 ml 1 M Tris–HCl, pH 8.5, 0.2 ml 1 M KCl, 30 μl of 1 M $MgCl_2$, 0.27 ml of water, BSA 5 mg); 2.3 μl of dNTP (2.5 mM each of dATP, dTTP, dGTP, and 1.7 mM of dCTP, Invitrogen, Carlsbad, CA); 1.2 μl of 1 mM of CY5-dCTP (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA); 1.2 μl of primers Amp-F and Amp-R (50 ng/μl each); 0.3 μl of Taq DNA polymerase (5 unit/μl, Invitrogen, Carlsbad, CA); and 2 μl of bacterial DNA or fecal DNA (1–10 ng/μl). The Amp-F and Amp-R primer sequences are GAGAGTTTGATYCTGGCTCAG and AAGGAGGTGATCCARCCGCA,

Table 1
Bacterial name* and numbers and the corresponding probe numbers and sequences

Bacterial		The corresponding probe	
Number	Species and strain	Number	Sequences
1	*Bacteroides thetaiotomicron* ATCC 29148	1	Aatacccgatggtataatcagaccgcatggtttgttatta
		2	Cattaggcagttggtgaggtaacggctcaccaaaccttcg
		3	Cagctacctggtgacaggatgctaatcccaaaagcctctc
2	*B. vulgatus* ATCC 8482	4	Aaggaataaagtcgggtatggatacccgtttgcatgtactt
		5	Agatgaattacggtgaaagccgtaagccgcaaggcatctg
		6	Tgttgtcagttactaacaggttatgctgaggactctgaca
3	*B. fragilis* ATCC 23745	7	Ccgatagcataatgattccgcatggtttcattattaaagg
		8	Tcgtaaacttcttttatataagaataaagtgcagtatgta
		9	Gaaggcagctagcgggtgaccgtatgctaatcccaaaat
4	*B. distasonis* ATCC 8503	10	Gcgggacgtgtcccgttttgtatgtaccttatgaataagg
		11	Ttcggaccgaggtggaaacacctttttctagcaatagccgt
		12	Aggccacctggcgacagggagcgaatccccaaaccacg
5	*Clostridium clostridioforme* ATCC 29048	13	Agtgccgcatggcagtgtgtgaaaaactccggtagtgtga
		14	Gaagcaagtctgaagtgaaaacccagggctcaaccctggc
		15	Cccctgacggccggtaacgcggccnttcttcgggacaggg
6	*C. leptum* ATCC 29065	16	Ctctgttcttagtgacgataatgacggtagctaaggagaa
		17	Tctatgggcttaacccataaactgcgcttgaaactgtctt
		18	Caaagccgcgaggtggagcaaaaccctaaaagcagtcc
7	*Fusobacterium prausnitzii* ATCC 27768*	19	Gtcgaacgagcgagagaggagcttgctttctcaagcgagt
		20	Cctgcgacgcgcatagaaatatgtgtttcttcgggaccag
		21	Gagaagcaagaccgcgaggcgagcaaaactcagaaacttcg
8	*Peptostreptococcuss productus* ATCC 27340*	22	Taagacggatttcttcggattgaagtctttgtgactgagc
		23	Ggaagagcaagtctgatgtgaaaggctggggcttaacccca
		24	cctctgaccgtc ccgtaacggg ganttccctt cggggcaga
9	*Ruminococcus obeum* ATCC 29174	25	Aacctttcattgaagcttcggcagatttggtctgtttcta
		26	Gtcccttaaccggatctttccttcgggacaggggagacag
		27	Cctatccccagtagccagcagtccggctgggcactctgag
10	*R. bromii* ATCC 27255	28	Gaatgctaataccgcatgacatatcggaaccacatggttc
		29	Cttcttttattaaggacgaaaaatgacggtacttaatga
		30	Taatacccgaagtcagtagtccaacctcgtgaggacgctg
11	*R. callidus* ATCC 27760	31	Catggattcgcatgtttctgtgatcaaagatttatcgcttaga
		32	Tgaagaggacgataatgacggtactcttttagaaagctc
		33	Aaagccggtcgtctaaccttcgggaggatgccgtctaagg
12	*R. albus* ATCC 27210	34	Ccaattggaaacgattgttaatacctcataacataacgaat
		35	Agagggaagcaaaacagtgatgtggagcaaaacccta
		36	Cctgtgttctaaccgcaaggaggaagcagtcgaaggtgg
13	*Bifidobacterium longum* ATCC 15687	37	Cttgatggcggggtaacggcccaccatggctttgacgggt
		38	Ggcttgacatgttcccgacgatcccagagatggggtttcc
		39	Agccggtggcctaacccctgcgggagggagccgtctaatg
14	*B. adolescentis* ATCC 15703	40	Ggatcggctggagcttgctccggccgtgagagtggcgaa
		41	Ctccagttggatgcatgtccttctgggaaagattctatcggt
		42	Caacgggatgcgacctcgtgaggggagcggatccctt
15	*B. infantis* ATCC 15697	43	Tcgaacgggatccatcgggctttgcttggtggtgagagtg
		44	Ccagttgatcgcatggtcttctgggaaagctttcgcggta
		45	Caacgggatgcgacgcggcgacgcggagcggatccctga
16	*Eubacterium biforme* ATCC 27806	46	Aagagaaaaacgacattcatagggaatgatgagtgagtgat
		47	Gtgatatgttactaacattgagttgaggactcatatcaga
		48	Agagcggcaagcctgtgaaggcaagcgaatctcataaagga
17	*E. aerofaciens* ATCC 25986*	49	Gcccgaaaggacgggtaataccggataccccggggtgc
		50	Cggcaggccgggggtcgaagcgggggctcaaccccccgaa
		51	Atgggtgaagcgggggagacccgtggccgagaggagcccata
18	*Lactobacillus acidophilus* ATCC 4356	52	Tgaaccaacagattcacttcggtgatgacgttgggaaacgct
		53	Gcaatccgtagagatacggagttcccttcggggacacta
		54	Acagtacaacgaggagcaagcctgcgaaggcaagcg
19	*Escherichia coli* ATCC 25922	55	Acaggaagaagcttgctctttgctgacgagtggcgga
		56	Ggaagggagtaaagttaataccttttgctcattgacgttac
		57	Catccacggaagttttcagagatgagaatgtgccttcgg
20	*Enterococcus faecium* ATCC 19434	58	Tgatttgaaaggcgctttcgggtgtcgctgatggatggac
		59	Gaagaacaaggatgagagtaactgttcatcccttgacgg
		60	Gaagtacaacgagttgcgaagtcgcgaggctaagctaat

(*continued on next page*)

Table 1 (*continued*)

Bacterial		The corresponding probe	
Number	Species and strain	Number	Sequences
21	*Bacteroides uniformis* ATCC 8492	61	Gcatgaacttagcttgctaagtttgatggcgaccggcg
		62	Atggcatagttcttccgcatggtagaaactattaaagaa
		63	Acgggaataaagtgaggcacgtgtgccttttgtatgtac
22	*B. ovatus* ATCC 8483	64	Tagtttgttggcggggtaacggcccaccaagactacgatg
		65	Ggtcaatgggcgagagcctgaaccagccaagtagcgtg
		66	Caacagaatatattggaaacagtatagccgtaaggctgt
23	*B. caccae* ATCC 43185	67	Gaaagattaatatccgatagcatatatttcccgcatggg
		68	Aagtggtccacgtgtggacttttgtatgtaccatatgaat
		69	Aatgaattatggggaaacccatacgccgcaaggcatntg
24	*Clostridium perfringens* ATCC 13124	70	Gaaggttttcggatcgtaaagctctgtctttggggaagat
		71	Tgcattactcttaatcgaggaaatcccttcggggacaagg
		72	Caacgagcgcaacccttgtcgttagttactaccattaagt
25	*C. butyricum* ATCC 19398	73	Gcataagattgtagtaccgcatggtacagcaattaaagg
		74	Tactctgtaatggaggaagccacttcggtggcaggaaga
		75	Tcggtacaatgagatgcaacctcgcgagagtgagcaaaa
26	*C. ramosum* ATCC 25582	76	Tgcctcaaagcactggtagaggatggacttatggcgcat
		77	Aagaagaacggcggctacaggaaatggtagccgagtga
		78	Actcataaaggctccagagatggagagatagctatatga
27	*C. difficile* ATCC 9689	79	Ttgccaagccgtaaggtggagctaatcccttaaagctac
		80	Atgctaatacgggataatatatttgagaggcatctcttga
		81	gcttgacatcccaatgacatct ccttaatcggagagttcc
28	*C. indolis* ATCC 25771	82	Gacggcgatgcaagtctggagtgaaagcccggggctcaac
		83	Gaccggtccgtaacggggccttcccttcggggcattccag
		84	Aacaaagggaagcaaaggagtgatctggagcaaaccccaa
29	*Fusobacterium russii* ATCC 25533	85	Tcattgcatgatgaagtcatgaaagctataagcgctgtga
		86	Taagggctcagagatgagcttgtgctccttcgggagaaag
		87	Gaacagagagtggcgaagctgtgaagtggagcaaatctc
30	*F. nucleatum* ATCC 25586	88	Tctacttgaatttgggttttttaacttcgatttgggtggcg
		89	Tgatattatgattatagggcatcctagaattatgaaagct
		90	Aggaatgagacagagatgtttcagtgtcccttcggggaa
31	*Bifidobacterium catenulatum* ATCC 27539	91	Ggtagtcggcggggtaacggcccaccgagcctacgacg
		92	Atgccggatgctccgactcctcgcatggggtgtcgggaa
		93	Gacatgttcccgacagccgtagagatacggtctcccttc
32	*B. angulatum* ATCC 27535	94	Tcgaacgggatcggctggagcttgctccggccgtgagag
		95	Tgctccagtccatcgcatggtggtctgggaaagattttat
		96	Acatgttcccgacagccccagagatggggcctcccttcgg
33	*Eubacterium rectale* ATCC 33656	97	Agcactttatttgatttccttcgggactgattattttgtg
		98	Ccttctgaccggtacttaaccgtaccttctcttcggagcag
		99	Gtaaacaaagggaagcaaagctgtgaagccgagcaaatc
34	*E. eligens* ATCC 27750	100	Gcatttacgaacagattatttcggtatgaagttcctttatg
		101	Cttgtactgggggatagcagctggaaacggctggtaatac
		102	Cgcacaatgttgcatgacatggtgtgaaaaactccggtgg
35	*E. limosum* ATCC 8486	103	Ggttttgaatgatccttcgggtgaaattagaactggaaag
		104	Ttatggttttgtcgcatggcgagatcatgaaaactccggtg
		105	Ctgacgagcctagagataggaagtttccttcgggaacaga
36	*E. lentum* ATCC 25559*	106	Aggtcgagcgatgaaaccgccctcgggcggacatgaag
		107	Tgctccggacaaccttgggaaaccgaggctaatatccgca
		108	Gacgtgaagccggggaaacccggtggctgagaggagcgt
37	*Lactobacillus fermentum* ATCC 9338	109	Attgattgatggtgcttgcacctgattgattttggtcgcca
		110	Aacaacgttgttcgcatgaacaacgcttaaaagatggctt
		111	Tgttaaagaagaacacgtatgagagtaactgttcatacgt
38	*Enterococcus faecalis* ATCC 27274	112	Tgccgcatggcataagagtgaaaggcgctttcgggtgtc
		113	Caaggacgttagtaactgaacgtcccctgacggtatctaa
		114	Gaagtacaacgagtcgttagaccgcgaggtcatgcaaatc
39	*Peptostreptococcus magnus* ATCC 14955*	115	Cgcgtggacaacctgcctatgacagtgggatagcctcggg
		116	Gtttaataagtcgaatgttaaagatcggggctcaaccccg
		117	Agcattggaaactgataaacttgagtagtggagaggaaa
40	*Ruminococcus gnavus* ATCC 29149	118	Agcaccttgacggatttcttcggattgaagccttggtgac
		119	Gcataagcgcacagtaccgcatggtacggtgtgaaaaac
		120	Aactgtcaggctagagtgtcggagaggaaagcggaattc

Some bacteria have been re-classified as indicated by * : *Faecalibacterium prausnitzii* (*Fusobacterium prausnitzii*) [7], *Ruminococcus productus* (*Peptostreptococcus productus*) [8], *Collinsella aerofaciens* (*Eubacterium aerofaciens*) [9], *Eggerthella lenta* (*Eubacterium lentum*) [11], *Finegoldia magna* (*Peptostreptococcus magnus*) [10].

respectively (Y is C or T; R is A or G). The use of these two primers for near full length (1.5 kb) amplification of 16S rRNA gene has been previously reported [41].

PCR was performed in a 9700 GeneAmp PCR System (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA), using thin-walled 0.2 ml tubes. The amplification conditions were incubation at 95 °C for 3 min, then 35 cycles of 95 °C for 10 s, 53 °C for 10 s, and 72 °C for 70 s, followed by one cycle at 72 °C for 4 min and a cool down to 4 °C.

The PCR product was purified with a Centri-Spin column (Princeton Separations, Adelphia, NJ) following the manufacturer's instructions. The purified CY5-labeled PCR products were then dried by Speed-Vac centrifugation (Savant, Farmingdale, NY).

2.4. Genomic DNA isolation from fecal samples and bacteria for PCR

Human fecal samples were collected from 11 healthy individuals (14–55 years old). One gram (wet weight) of fresh fecal sample was mixed with 9 ml of 0.85% NaCl in a 15 ml tube, then centrifuged at a low speed (300*g*) for 10 min to remove large particles. This centrifugation–washing step was repeated two times, then the upper phase were centrifuged at 7000*g* for 10 min to collect the pellets. The aspirated pellets were transferred to 1.5 ml tubes. The pellet was suspended in 0.8 ml ddH_2O, mixed, then centrifuged at 16,000*g* for 5 min to collect the pellets. The pellets were mixed with 350 μl of solution A (Easy-DNA kit, Invitrogen, Carlsbad, CA), and heated at 75 °C for 20 min. A 150 μl of solution B (from Easy-DNA kit) was added to the reaction mixture, mixed well, followed by the addition of 500 μl of chloroform with mixing. The tube was centrifuged for 10–20 min at 16,000*g*. The upper phase was transferred to a new tube and 1 ml 100% ethanol was added and mixed. After cooling the tube at −20 °C for 10 min, the tube was centrifuged at 16,000*g* for 10 min. The supernatant was discarded and the pellet was washed once with 1 ml 70% ethanol. The pellet was air-dried and then resuspended in 100 μl of sterile water. RNase A (5 μl × 2 mg/ml) was added to the solution, and the mixture was heated at 45 °C for 15 min. TE buffer (300 μl) (10 mM Tris pH 8.0, 1 mM EDTA) was added to dilute the DNA. The DNA concentration was determined by agarose gel electrophoresis with DNA standards. An aliquot of the DNA was diluted with 1% Triton X-100 to 10 ng/μl and used for PCR amplification.

Bacterial DNA for each of the bacterial species tested was isolated from 5 ml pure culture by centrifugation at 7000*g* for 10 min, followed by extraction using the Easy DNA kit. For experiments requiring an accurate determination of the bacterial cell numbers, the cells were counted using a Petroff–Hausser counting chamber (Hausser Scientific, Horsham, PA) and a phase contrast microscope [42].

2.5. Hybridization

The epoxy slides containing the array do not need prehybridization or blocking. The dried, purified, CY5-labeled PCR products were dissolved in 13 μl of MWG-biotech hybridization buffer. The tube was heated for 3 min in a boiling water bath, then immediately placed into ice water for 2 min. The solution was collected by brief centrifugation and applied onto the oligo area on the microarray slides. A small glass cover slip that was autoclaved and dried was used to cover the hybridization solution on the array area. The slide was placed into a hybridization chamber (Corning Inc., Corning, NY) and then immersed in a water bath for hybridization overnight at 42 °C.

After hybridization, the cover slip was removed by washing the slides for 5 min with 0.5 × SSC, 0.1% SDS. The slides were then washed for 5 min with 0.1 × SSC, 0.1% SDS, followed by a 5 min wash with 0.1 × SSC only. All the washing steps were conducted at room temperature. The slides were dried by centrifugation for 1 min at 3000*g* in an IEC clinical centrifuge with IEC CAT 801 rotor (International Equipment Company, Needham Heights, MA). The slides were kept in the dark at room temperature until scanned with the ScanArray Express Microarray Scanner (Packard BioScience-Perkin Elmer, USA). Potential cross-reactivity problems for the species-specific probes were resolved by designing three probes for each species. The abilities of the probes to hybridize to their target bacterial species were tested individually by hybridizing PCR products amplified from each isolated bacterial strain to the oligonucleotides spotted onto nitrocellulose membranes under the conditions previously described [40]. These positive reactions were then confirmed using the full array and PCR products from pooled DNA. Due to potential cross-reaction and inhibition of hybridization of individual probes when the array was hybridized with PCR products from the complex mixture of bacteria in the fecal samples, positive reactions for two or three of the probes for a species were required to consider an identification of a species as positive.

3. Results

Using the sequences of the 16S rRNA genes, 120 oligonucleotide probes were designed that are species-specific to 40 predominant bacterial species from the human intestine (three probes per species). With a few exceptions, each oligo has at least 3–5 nucleotide differences with the other 16S rRNA gene sequences. The probe numbers, sequences, and the target bacterial species are listed in Table 1. To demonstrate that the probes were capable of hybridizing to all the 40 species, the microarray was hybridized with CY5-labeled PCR products amplified from a mixture of DNA extracted from purified cultures of all 40 bacteria (Fig. 1). Although the spots differ in intensity,

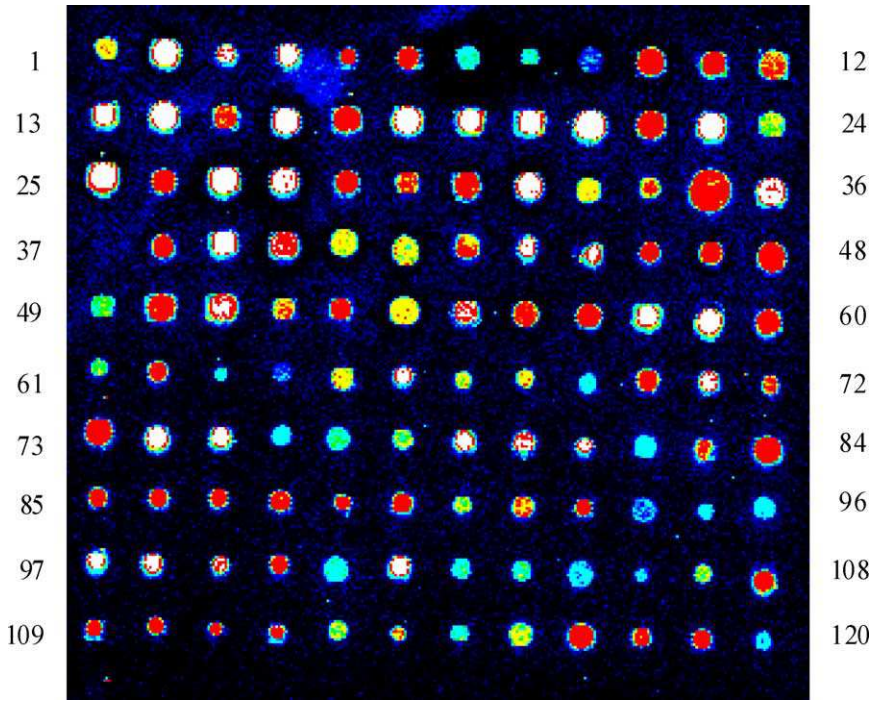

Fig. 1. Microarray results for DNA mixture of the 40 reference bacterial species. The probe location starts at 1, 2, 3, … and goes until 120; with 12 probes per line and 10 lines on all microarray slides. The probe numbers with the corresponding bacterial species are listed in Table 1. The intensities of signal from high to low are white (saturated), then red, yellow, green, and blue (see web version). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

all probes were positive with the exception of #37. Subsequent experiments indicated that this probe was capable of hybridizing to the target bacterium (*Bifidobacterium longum*), and that its absence in this experiment may have been due to competitive inhibition in the 40 DNA mixture.

3.1. Sensitivity and specificity test for the microarray method

Two methods were conducted for the sensitivity test: we determined the cell number limitation for the microarray method and compared the microarray method with culture method by using the identical fecal sample. When the microarray method was used for detection of bacteria in fecal samples, the total DNA isolated from fecal samples was used for PCR amplification—labeling, and then hybridization. If using DNA concentration as an indicator for microarray sensitivity, it was difficult to convert to cell numbers. So, we directly used bacterial cells to do PCR amplification and labeling, and then do hybridization. We made pure culture for 22 species, the species numbers are 1, 3, 4, 5, 6, 8, 9, 10, 12, 13, 18, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30 and 33 (Table 1). The cell numbers were counted with a phase-contrast microscope [42]. A cell mixture of the 22 species was made for 10^5 cells/2 μl for each species. A series dilution of the cell mixture with ddH_2O was made to 10^4, 10^3, 10^2, 10 cells/2 μl for each species. The cell mixtures (2 μl each) were directly used for PCR amplification and labeling as described in Section 2. Fig. 2 shows the results of the microarray test for 10^5 and 10 cells each species of 22 species. For the 10^5 cells, all 22 species gave positive results. Some cross-reactions were found (probes 6, 19, 21, 32, 40, 48, 49, 100 in Fig. 2). In general with a few exceptions, however, these cross-reactions were weak or only showed positive in one probe from the three probes for each species. However, the 16S rDNA sequences for different strains of *Bifidobacterium infantis* were different in the GenBank database; some of them were similar to some 16S rDNA sequences of *B. longum*. On the other hand, the 16S rDNA sequences for different strains of *B. longum* were also different; some of them were similar to 16S rDNA sequences of some *B. infantis*. Actually, these two species are close-related *Bifidobacterium* and many authors described them as *B. longum* bv. *Infantis* (in GenBank). Therefore, some cross-reaction may not be necessarily correct but due to similar bacterial strains that were given wrong species name in the GenBank. The cross-reaction problem was mostly resolved by designing three probes for each species. At least two probes shown positive for one species were considered as positive for this species.

For the 10 cells, about nine species (species numbers 3, 4, 5, 8, 20, 25, 27, 28, 29 in Fig. 2) from 22 species showed positive, which indicated that the sensitivity of this method could be reach to 10 cells for these nine species. However, since these results were obtained from direct cell-PCR without isolation of the DNA, the results may be different from the DNA-isolation-PCR method.

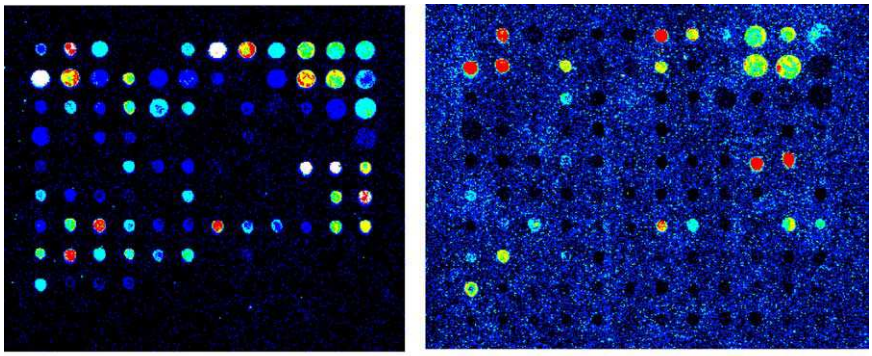

Fig. 2. Microarray test results for 10^5 and 10 cells each species of 22 species (species numbers are 1, 3, 4, 5, 6, 8, 9, 10, 12, 13, 18, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 33). The species names with the numbers are listed in Table 1.

3.2. Comparison of different methods for isolation of DNA from fecal samples

Identification of bacteria from fecal samples using the microarray method requires efficient and non-selective extraction of DNA. McOrist et al. [43] compared the relative efficacy of extraction of bacterial DNA (both Gram negative and positive origin) from feces using four commercial kits (FastDNA kit, Bio 101; Nucleospin C + T kit, Macherey-Nagal; Quantum Prep Aquapure Genomic DNA isolation kit, Bio-Rad; and QIAamp DNA stool mini kit, Qiagen) and a non-commercial guanidium isothiocyanate/silica matrix method. They indicated that for fecal samples, the Qiagen QIAamp DNA stool mini kit was the most effective extraction method. We compared the Qiagen kit with the Invitrogen Easy-DNA kit for fresh or frozen fecal samples and found that the modified Easy-DNA procedure (as described in Section 2) gave slightly better results. Amplification from DNA prepared using the modified EASY-DNA method gave improved signals over the Qiagen method on about 10 probes when fresh fecal samples were used (Fig. 3). When frozen fecal samples were used, DNA prepared using the modified Easy DNA method resulted in lost or weaker signals on only about four or five probes when compared to DNA isolated from fresh fecal samples (Fig. 3).

In general, the efficiency of recovery of DNA from G + bacterial species is lower than that from G − bacterial species. For example, the efficiency of recovery of DNA from G − bacterial species is 80–100%, however,

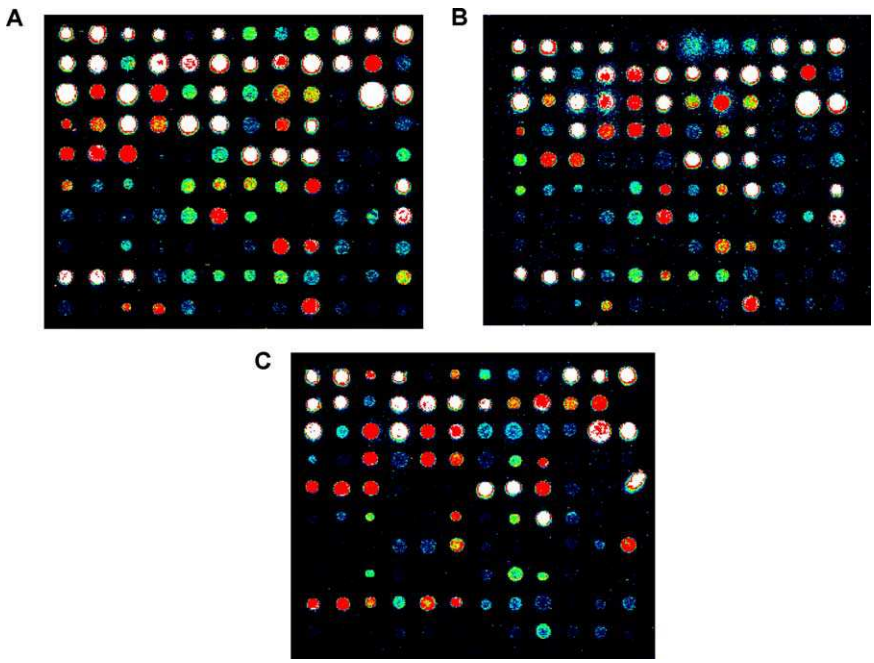

Fig. 3. Comparison of microarray test results, using different DNA isolation methods from fresh and frozen fecal samples for a same individual. Panel A, Washing-Easy DNA kit method from fresh fecal sample. Panel B, Washing-Easy DNA kit method from frozen fecal sample. Panel C, Qiagen QIAamp DNA stool mini kit method from frozen sample.

the efficiency of recovery of DNA from G + bacterial species is only 1–20% (data not shown). However, if the bacterial cell number in fecal sample was 10^9 per gram fecal sample, the microarray test results will still be positive even if the efficiency of recovery of DNA is 1%. So the lower efficiency of DNA recovery will only affect the bacterial species present with lower numbers in fecal samples.

3.3. Comparison of the microarray method with standard culture techniques

The microarray method was compared with standard culture methods for the identification of bacteria from the same fecal sample. Using standard selective and non-selective culture techniques as described in Section 2, 55 colonies were selected and purified for identification. Forty-three isolates were identified to the species level, representing 18 distinct bacterial species. Using the microarray, 21 of the 40 species in the array were scored as positive in the same fecal sample. Eleven species, including *Bacteroides caccae*, *B. distasonis*, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *Bifidobacterium adolescentis*, *Clostridium clostridioforme*, *Collinsella aerofaciens*, *Eubacterium rectale*, *Ruminococcus productus*, and *R. callidus*, were identified by both the culture and microarray methods. Ten species, *Bacteroides ovatus*, *B. uniformis*, *B. infantis*, *B. longum*, *Clostridium leptum*, *Eubacterium eligens*, *Faecalibacterium prausnitzii*, *Ruminococcus albus*, *R. bromii*, and *R. obeum*, were positive in the microarray but not identified by culture. Six species, *Bifidobacterium dentis*, *Clostridium innocuum*, *Coprococcus comes*, *Streptococcus anginosus*, *S. intermedius*, and *S. oralis*, were positive by culture but not included in the microarray. Only one species, *E. coli*, was positive by culture and negative by the microarray for this fecal sample. This result is probably due to the fact that plating on MacConkey medium provides a strong selection for *E. coli*. This allowed the detection of *E. coli* at 3.7×10^4 CFU/g, which is below the 10^5–10^6 CFU/g detection limit for the microarray method (data not shown). The BBE medium is highly selective for *Bacteroides* species, which are among the most prevalent intestinal bacteria. The different *Bacteroides* species detected by plating are probably present in the fecal sample in roughly the same amounts (within an order of magnitude). It is possible that *B. uniformis* and *B. ovatus* were present in this fecal sample in slightly lower numbers than the five *Bacteroides* species that were isolated, and screening of a larger number of isolates might detect them. The culturing methods that were used did not give a strong selection for any of the other bacterial species identified by the microarray assay, but screening of a large number of additional colonies on the non-selective media might have resulted in identification of more of the microarray-positive species.

3.4. Detection of the 40 bacterial species from fecal samples obtained from 11 healthy human volunteers

The microarray method was used to detect the 40 bacterial species from fecal samples obtained from 11 healthy human volunteers. Fig. 4 and Table 2 show the microarray results for all 11 fecal samples. The intensities of

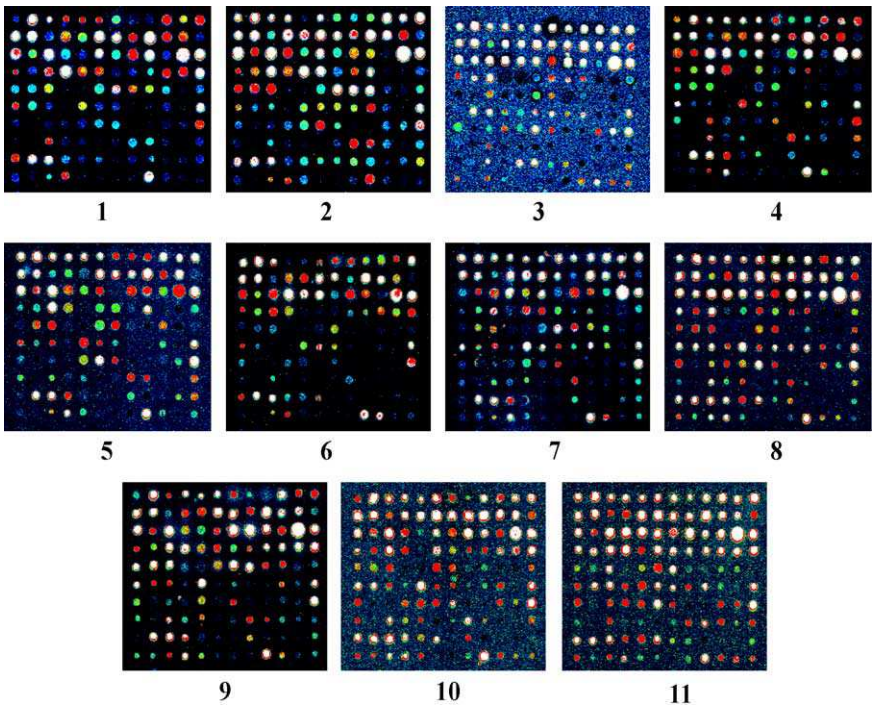

Fig. 4. Microarray results for 11 fecal samples. The results are translated into Table 2.

Table 2
Microarray test results read from Fig. 4 for fecal samples obtained from 11 healthy human volunteers

Bacterial species	Human volunteer's numbers for fecal samples										
	1	2	3	4	5	6	7	8	9	10	11
Bacteroides thetaiotaomicron	+	+	+	+	+	+	+	+	+	+	+
B. vulgatus	+	+	+	+	+	+	+	+	+	+	+
B. fragilis	−	+	+	+	+	+	+	+	+	+	+
B. distasonis	+	+	+	+	+	+	+	+	+	+	+
B. uniformis	+	+	+	+	+	+	+	+	+	+	+
B. ovatus	+	+	+	+	+	+	+	+	−	+	+
B. caccae	−	+	+	+	−	+	+	+	−	+	+
Clostridium clostridioforme	+	+	+	+	+	+	+	+	+	+	+
C. leptum +	+	+	+	+	+	+	+	+	+	+	
C. perfringens	+	+	−	+	+	+	+	+	+	+	+
C. butyricum	+	+	+	+	+	−	−	+	+	−	+
C. ramosum	+	+	+	+	+	+	+	+	−	+	+
C. difficile	−	−	−	−	−	−	−	−	−	−	−
C. indolis	+	+	+	+	+	−	+	+	+	+	+
Ruminococcus obeum	+	+	+	+	+	+	+	+	+	+	+
R. bromii	+	+	+	+	+	+	+	+	+	+	+
R. callidus	+	+	+	+	+	+	+	+	+	+	+
R. albus	+	+	+	+	+	+	+	+	+	+	+
R. gnavus	−	−	+	−	+	+	+	+	+	+	+
R. productus *	+	+	+	+	+	+	+	+	+	+	+
Faecalibacterium prausnitzii *	+	+	+	+	+	+	+	+	+	+	+
Bifidobacterium longum	+	+	+	+	+	+	+	+	+	+	+
B. adolescentis	+	+	−	+	+	−	+	+	+	+	+
B. infantis	+	+	+	+	+	+	+	+	+	+	+
B. catenulatum	+	+	−	+	+	−	−	+	+	+	+
B. angulatum	−	+	−	+	−	−	−	−	+	−	+
Eubacterium biforme	+	−	−	−	−	+	−	−	+	+	+
E. rectale	+	+	−	+	+	+	+	+	+	+	+
E. eligens	+	+	+	+	−	+	+	+	−	+	+
E. limosum	−	+	+	−	−	−	−	+	−	−	+
Eggerthella lenta *	−	+	−	−	+	−	+	−	−	−	−
Collinsella aerofaciens *	+	+	−	+	+	+	+	+	+	+	+
Fusobacterium russii	+	+	−	−	+	+	+	+	+	+	+
F. nucleatum	−	−	−	+	−	−	+	+	+	+	−
Finegoldia magna *	−	+	−	−	−	−	−	+	−	+	+
Lactobacillus acidophilus	−	−	−	−	−	−	−	−	+	−	+
L. fermentum	+	+	+	−	−	−	+	+	+	+	+
Enterococcus faecium	+	−	−	−	−	−	−	−	+	−	+
E. faecalis	−	+	−	−	+	−	+	+	+	+	+
Escherichia coli	+	+	+	−	−	−	+	+	+	+	+
Total (+)	29	34	25	28	28	25	31	34	32	33	37

Some bacteria have been re-classified as indicated by *: *Faecalibacterium prausnitzii* (*Fusobacterium prausnitzii*) [7], *Ruminococcus productus* (*Peptostreptococcus productus*) [8], *Collinsella aerofaciens* (*Eubacterium aerofaciens*) [9], *Eggerthella lenta* (*Eubacterium lentum*) [11], *Finegoldia magna* (*Peptostreptococcus magnus*) [10].

signal from high to low are white (saturated), then red, yellow, green, and blue (see web version) (Fig. 4). If two or three probes for one species gave a positive signal, the sample was considered positive (+) for this species. If only one probe for one species showed a positive signal, then the sample was considered negative (−) for this species (Table 2). The results indicated that 25–37 species could be detected from each fecal sample by the microarray, and that 39 of the 40 species were present in two or more different samples. Only *Clostridium difficile*, which is a pathogen [44], was negative in all 11 fecal samples (Table 2). The intestinal bacteria found in the majority of the fecal samples by using the microarray method were the 33 species indicated below: *Bacteroides thetaiotaomicron*, *B. vulgatus*, *B. fragilis*, *B. distasonis*, *B. uniformis*, *B. ovatus*, *B. caccae*, *Clostridium clostridioforme*, *C. leptum*, *C. ramosum*, *C. indolis*, *C. perfringens*, *C. butyricum*, *Faecalibacterium prausnitzii*, *Fusobacterium russii*, *F. nucleatum*, *Ruminococcus productus*, *R. obeum*, *R. bromii*, *R. callidus*, *R. albus*, *R. gnavus*, *B. longum*, *B. adolescentis*, *B. infantis*, *B. catenulatum*, *Eubacterium biforme*, *E. rectale*, *E. eligens*, *Collinsella aerofaciens*,

Lactobacillus fermentum, *Enterococcus faecalis*, and *Escherichia coli*. There were no two fecal samples that gave exactly the same profile of bacterial species (Table 2).

4. Discussion

Since intestinal bacteria play an important role in human physiology, numerous papers have been published using traditional culture and molecular methods to detect and identify them in fecal samples. Culture methods are time consuming and many intestinal bacteria are not easily cultured or isolated from the complex mixture. Moreover, many molecular methods are group-specific for bacteria, but not species-specific [22,27,45–49], as described by Matsuki et al. [48]: "the complex microflora of the human gut is difficult to study with only primers that are specific at the species level due to the diversity of this ecosystem." Several papers reported detection of intestinal bacteria on the species level; however, the number of species detected was limited [25,32–34,50].

In this study, we described a microarray method for the detection and identification of 40 intestinal bacterial species on one slide, with the potential to test many more. The 40 intestinal bacterial species tested include seven species each of *Bacteroides* and *Clostridium*, six species of *Ruminococcus*, five species of *Bifidobacterium*, four species of *Eubacterium*, two species each of *Fusobacterium*, *Lactobacillus* and *Enterococcus*, and single species each of *Collinsella*, *Eggerthella*, *Escherichia*, *Faecalibacterium* and *Finegoldia*. The majority of these species were identified in the published literature [1–6] as organisms that are predominant in the human colon. Since most prior species identification was done using culture techniques, this group of bacteria favors organisms that can be cultured, and may not include some major fecal components that are less readily isolated using standard culture techniques. As additional major bacterial species are identified by culture or molecular methods, probes to detect them can be easily added to this type of array.

The results from this study indicated that the microarray method is more sensitive than culture methods for bacterial species for which there is no strong selection method. The current detection time is 24 h (plus microarray preparation and sample preparation time), but it could be reduced with new techniques that can reduce the hybridization time.

In some methods that require extensive PCR amplifications, errors accumulated during the amplification can influence the outcome. Also, in methods where multiple DNA targets are amplified, a bias toward some templates over others can substantially affect the relative levels of the PCR products. We chose 35 PCR cycles for amplification of all bacterial DNA present in fecal samples in order to enhance the detection of rare members of the population, but this came at the expense of being able to quantify the proportion of each bacterial group in the whole population. Because the large number of PCR cycles increase the potential for PCR bias and chimera formation, our microarray assay is qualitative rather than quantitative.

Using this microarray method we were able to establish profiles of predominant fecal bacteria from several different healthy individuals. Many *Bacteroides*, *Clostridium*, and *Ruminococcus* species were confirmed as present in all the samples. *Bifidobacterium* and *Eubacterium* species were more varied in their distribution among the different individuals. This method should be useful for monitoring changes in the intestinal population due to normal variation, or in response to outside agents. Further development of this assay and expansion to additional bacteria will extend its usefulness in many area of intestinal microbiology.

In conclusion, an oligonucleotide–microarray method was developed for the detection of 40 predominant intestinal bacterial species from human fecal samples. The microarray method can detect most of these bacteria in human fecal samples, including 33 species that are positive in most of the fecal samples and 39 species in at least two samples. However, some unique differences in bacterial species were found in the fecal samples from each individual.

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Exhibit 415

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DNA Sequencing Says New Hybrid E. Coli Strain Is Cause Of German Outbreak

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Preliminary data from DNA sequencing performed in cooperation with the University Hospital Muenster, Germany, on the Ion Personal Genome Machine (PGM) strongly suggests that the bacterium at the root of the deadly outbreak in Germany is a new hybrid type of pathogenic *E. coli* strains.

The data obtained from the DNA sequencer shows the presence of genes typically found in two different types of *E. coli*: enteroaggregative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC). These results, which are being confirmed by further data analysis on the Ion PGM(TM), may provide insight into this bacterium's aggressiveness and help prevent further outbreaks.

The data gathered at Life Technologies' laboratories in Darmstadt, Germany, will be used by scientists at the University Hospital Muenster to develop better tests to positively identify the illness in people showing symptoms of the infection, which includes kidney failure and bloody diarrhea. For the first time since the outbreak, it is now possible to study in detail the basis for what makes this strain so aggressive.

"The rapid whole genome sequencing results enabled us to discover within days a unique combination of virulence traits ... and makes this German outbreak clone a unique hybrid of different *E. coli* pathovars," said Dr. med. Alexander Mellmann, scientist at the German National Consulting Laboratory for Hemolytic Uremic Syndrome (HUS) at the Institute of Hygiene, University Hospital Muenster.

Life Technologies' Simone Guenther, Ph.D., who carried out the sequencing work, said, "The severity of this outbreak meant that speed was of the essence. We were able to provide the data in record time to University Hospital Muenster. In previous outbreaks it would have taken much longer to reach this stage."

To help prevent further spreading of the bacterium, Life Technologies began shipping its *E. coli* testing kits to European laboratories to screen contaminated food thought to be at the center of the outbreak that has killed 17 people and affected more than 1,000 throughout Europe.

The kits are a first line of defense to detect the presence of pathogenic *E. coli* and then secondary testing is performed using more specific kits. Life Technologies will develop new customized kits specifically designed to detect the hybrid strain in Germany once the sequencing data has been fully analyzed in the next few days. The company can design custom assays in less than one week.

The kits shipped to Europe are part of a large offering of molecular tests developed by Life Technologies capable of accurately detecting most pathogens that are dangerous to humans. The Ion PGM(TM) is a DNA sequencing instrument featuring semiconductor technology that can complete a highly accurate sequence of DNA in just two hours. The DNA sample used for the sequencing work was collected from a patient with the illness.

The Ion Personal Genome Machine sequencer is for research use only and is not for use in diagnostic procedures - but you can take one with you to that organic farmer's market.

RELATED ARTICLES ON SCIENCE 2.0

E. Coli Strain Responsible For Food Poisoning Gets Its Genome Sequenced

Ion Torrent Ships New RNA Sequencing Application As Fast, Easy, Affordable Alternative To Microarrays

Deadly E. Coli Strain Sequenced With Roche GS Junior System By HPA Scientists - Provides Community Most Complete Assembly To Date

Life Technologies' Benchtop Ion Proton™ Sequencer Designed To Decode A Human Genome In One Day For $1,000 Makes ArabLab Debut

UM School Of Medicine Institute For Genome Sciences Cracks Genomic Code Of Deadly German E. Coli Outbreak

Exhibit 416

About ARUP

ARUP Laboratories is a national clinical and anatomic pathology reference laboratory and an enterprise of the University of Utah and its Department of Pathology. With more than 3,000 employees, ARUP offers in excess of 3,000 tests and test combinations, ranging from routine screening tests to highly esoteric molecular and genetic assays.

Philosophy | Clients | Technical Services | Trusted Pathologists

Philosophy

Rather than competing with its clients for physician office business, ARUP chooses instead to support clients' existing test menus by offering highly complex and unique lab tests, with accompanying consultative support, to enhance their abilities to provide laboratory services.

ARUP offers a comprehensive suite of services that enables clients to have a positive impact on their local health community. ARUP's Utilization Management Suite of Services includes:

- ARUP ATOP®—a laboratory analytics service designed to identify potential over-, under-, and mis-utilization of individual laboratory tests.
- ARUP Connect™—an online information system for referral test management; easily access test results, submit statistical data, and retrieve secure files.
- ARUP Consult®—a revolutionary guide, available on both PDA and Web platforms, designed to assist the clinician with laboratory test selection and interpretation.
- ARUP Direct™—a complete, start-to-finish outreach program designed to help every client, regardless of size or development stage.
- ARUP Gateway™—a uniquely integrated customer test menu tool, branded to serve the client's outreach medical community.
- ARUP Insource Advantage™—an analytical service that evaluates the economic feasibility of performing specific tests in-house (make) vs. sending these same tests to a reference laboratory partner (buy).

ILLUM-3929

Myeloid Malignancies Mutation Panel by Next Generation Sequencing

Indications for Ordering

Assess for single gene mutations, including substitutions and insertions and deletions that may have diagnostic, prognostic, and/or therapeutic significance in

- Acute myeloid leukemia
- Myelodysplastic syndromes
- Myeloproliferative neoplasms
- MDS/MPN overlap disorders such as chronic myelomonocytic leukemia

Test Description

- Next generation sequencing library construction from genomic DNA
- Enrichment for regions of interest by hybridization
- Massively parallel sequencing on Illumina platform

Tests to Consider

Primary test

Myeloid Malignancies Mutation Panel by Next Generation Sequencing 2011117

Related tests

- *CEBPA* Mutation Detection 2004247
- *NPM1* Mutation by PCR and Fragment Analysis 0040174
- *IDH1* and *IDH2* Mutation Analysis, exon 4 2006444
- *WT1* Mutation Detection by Sequencing 2005766
- *KIT* Mutations in AML by Fragment Analysis and Sequencing 2002437

Disease Overview

Diagnostic issues

- Genetic targets contained in this panel are relevant across the spectrum of myeloid malignancies
- Identification of one or more clonal genetic abnormalities may aid in establishing the diagnosis of a neoplasm
- Identification of certain mutations or patterns of mutations may aid in diagnostic subclassification

Prognostic and treatment issues

- Certain mutations or patterns of mutations may have prognostic significance.
- Certain mutations may allow for the use of targeted therapies

Genetics

Genes – *ASXL1, BCOR, BCORL1, BRAF, BRINP3, CALR, CBL, CEBPA, CSF3R, DNMT1, DNMT3A, EED, ETV6, EZH2, FLT3, GATA1, GATA2, HNRNPK, IDH1, IDH2, JAK2, JAK3, KDM6A, KIT, KMT2A, KRAS, LUC7L2, MPL, NOTCH1, NPM1, NRAS, NSD1, PHF6, PRPF40B, PTPN11, RAD21, RUNX1, SETBP1, SF1, SF3A1, SF3B1, SMC1A, SMC3, SRSF2, STAG2, SUZ12, TET1, TET2, TP53, U2AF1, U2AF2, WT1, ZRSR2*

Mutations

A full list of targeted regions within these genes can be found at the ARUP website – Myeloid Panel Coordinates

Test Interpretation

Sensitivity/specificity

- Analytical sensitivity – 5% mutant alleles (single nucleotide variants, insertions, and deletions)
- Analytical specificity – 100%

Results

- Positive – a somatic mutation in one of the 51 tested genes was detected
  - Clinical relevance (diagnosis, prognosis, therapy) will be correlated, if known
- Negative result – no mutations were detected in the sequenced genes

Limitations

- Mutations may be present below the limit of detection
- Not intended to detect minimal residual disease

Organism Identification by 16S rDNA Sequencing

0060720

Ordering Recommendation

ARUP Consult®
Disease Topics
Brucella Species
Tularemia
Endocarditis

Interface Map

Mnemonic

MC BACSEQ

Methodology

16S rDNA Sequencing

Performed

Sun-Sat

Reported

2-5 days

New York DOH Approval Status

This test is New York DOH approved.

Submit With Order

Specimen Required

Patient Preparation:

Collect: Actively growing isolated organism, in pure culture.

Specimen Preparation: Transport sealed container with pure culture on agar slant or in bacterial transport media.

Storage/Transport Temperature: Room temperature. If culture is suspected of being a microorganism listed as infectious substance affecting humans on IATA list, submit specimen according to Biological Substance, Category A, shipping guidelines.

Unacceptable Conditions: Mixed cultures or non-viable organisms.

Remarks: Specimen source required. Indicate suspected pathogen. For suspected agents of bioterrorism, *Salmonella*, or *Shigella*, notify your state department of health and refer isolates to your state laboratory for identification. For identification of Shiga-like toxin producing *E. coli,* order test *E. coli* Shiga-like toxin by EIA (ARUP test code 0060047).

Stability: Ambient: 1 week; Refrigerated: Unacceptable; Frozen: Unacceptable

Reference Interval

By report

Interpretive Data

See Compliance Statement B: www.aruplab.com/CS

Statement B: This test was developed and its performance characteristics determined by ARUP Laboratories. The U.S. Food and Drug Administration has not approved or cleared this test; however, FDA clearance or approval is not currently required for clinical use. The results are not intended to be used as the sole means for clinical diagnosis or patient management decisions.

Note

An additional processing fee will be billed for mixed cultures submitted as indicated in the specimen requirements. Identification and susceptibility tests are billed separately from culture.

CPT Code(s)

87153; Identification CPT codes may vary based on method.

Components

Component Test Code*	Component Chart Name	LOINC
0060720	Organism Identification by 16S rDNA	

** Component test codes cannot be used to order tests. The information provided here is not sufficient for interface builds; for a complete test mix, please click the sidebar link to access the Interface Map.*

Aliases

- 16S Ribosomal DNA (rDNA) Sequencing
- Bacterial sequencing

ILLUM-3932

Clostridium difficile toxin B gene (tcdB) by PCR

2002838

Ordering Recommendation

Recommended rapid, stand-alone diagnostic test for *C. difficile*-associated diarrhea.

ARUP Consult®
Disease Topics
Clostridium difficile
Malabsorption
IBD

Interface Map

Mnemonic

CDIFF PCR

Methodology

Qualitative Polymerase Chain Reaction

Performed

Sun-Sat

Reported

1-2 days

New York DOH Approval Status

This test is New York DOH approved.

Submit With Order

Specimen Required

Patient Preparation:

Collect: Soft or liquid stool.

Specimen Preparation: Transfer 1 mL stool to a clean, unpreserved transport vial (ARUP Supply# 40910). Available online through eSupply using ARUP Connect™ or contact ARUP Client Services at (800) 522-2787. (Min: 0.5 mL).

Storage/Transport Temperature: Refrigerated.

Unacceptable Conditions: Specimens in media or preservatives.

Remarks:

Stability: Ambient: 48 hours; Refrigerated: 5 days; Frozen: 1 month

Reference Interval

Not detected

Interpretive Data

Note

CPT Code(s)

87493

Components

Component Test Code*	Component Chart Name	LOINC
2002839	C. difficile toxin B test source	31208-2
2002840	C. difficile toxin B gene (tcdB), PCR	54067-4

** Component test codes cannot be used to order tests. The information provided here is not sufficient for interface builds; for a complete test mix, please click the sidebar link to access the Interface Map.*

Aliases

- C diff
- C. difficile PCR
- C. difficile PCR test
- C. difficile tcdB by PCR
- Cdiff

ILLUM-3934

Streptococcus Group B by PCR

0060705

Ordering Recommendation

ARUP Consult®
Disease Topics
Group B, Strep

Interface Map

Mnemonic

GBS PCR

Methodology

Qualitative Polymerase Chain Reaction

Performed

Sun-Sat

Reported

Within 48 hours

New York DOH Approval Status

This test is New York DOH approved.

Submit With Order

Specimen Required

Patient Preparation:

Collect: Rectal or vaginal swab.

Specimen Preparation: Transfer swab to Liquid Stuart media (ARUP supply: #43875). Available online through eSupply using ARUP Connect™ or contact ARUP Client Services at (800) 522-2787.

Storage/Transport Temperature: Refrigerated.

Unacceptable Conditions: Specimens in any transport media other than indicated above.

Remarks: Specimen source required.

Stability: Ambient: 24 hours; Refrigerated: 6 days; Frozen: Unacceptable

Reference Interval

Not Detected

Interpretive Data

For antibiotic sensitivity testing, contact ARUP Client Services at (800) 522-2787 and request Antimicrobial Susceptibility-Not Otherwise Specified (ARUP test code 0060200). Susceptibility testing must be ordered within 30 days of specimen collection.

Note

CPT Code(s)

87653

Components

Component Test Code*	Component Chart Name	LOINC
0060705	Streptococcus (Group B) by PCR	
2002869	Streptococcus (Group B) Source	

** Component test codes cannot be used to order tests. The information provided here is not sufficient for interface builds; for a complete test mix, please click the sidebar link to access the Interface Map.*

Aliases

- Beta-Hemolytic Streptococcus
- Beta-Hemolytic Streptococcus PCR

ILLUM-3936

Exhibit 417

About Us

Quest Diagnostics Nichols Institute of Valencia, founded in 1975, is a full-service, clinical reference laboratory focused on partnering with hospitals and commercial labs to support their role in community-based medicine and to reduce costs for episodes of care. Our lab remains a distinct operating division of Quest Diagnostics operating a 200,000 sq ft, state-of-the-art facility in Valencia, California as the center of excellence for Urology testing. Our lab will continue to provide cutting-edge research and development of new assays as well as refinement of existing diagnostic tests to produce assays with greater sensitivity, specificity, efficiency and clinical value for reliable and cost-effective patient assessment.

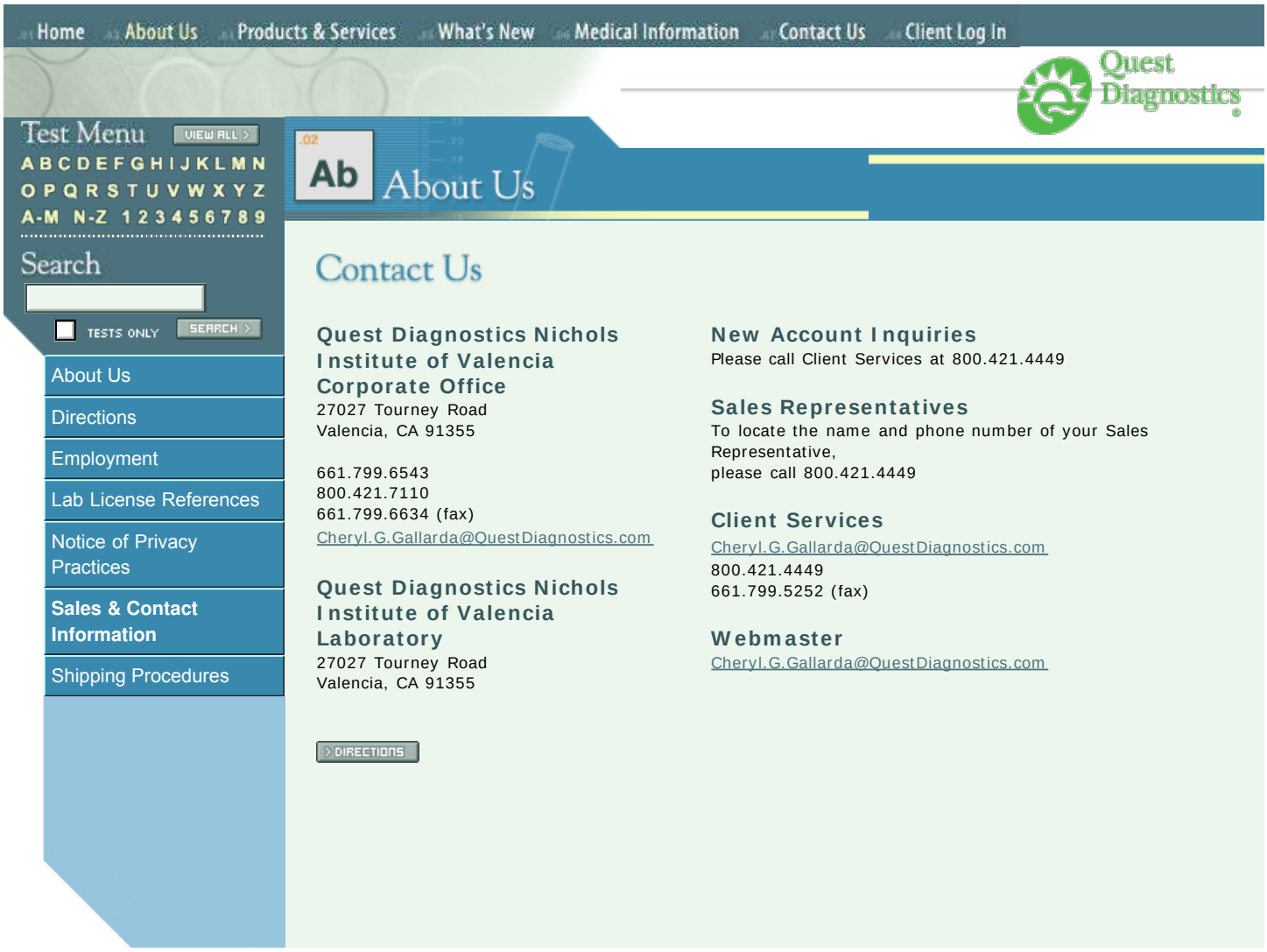
Our lab is uniquely positioned to more effectively support local pathology and community-based medicine for enhanced patient care. Our complementary areas of expertise and service offerings will allow us to build on both companies' leadership positions, provide access to each other's medical and scientific expertise, expand our geographic presence to better serve our customers, and become the most valued company in our industry.

TARO™: **T**OTAL **A**CCESSIONING **RE**-**O**RGANIZATION

Assays are available to support testing in a wide range of clinical areas, including:

Allergy & Immunology	Cardiology & Coagulation	Dermatopathology
Endocrinology	Gastroenterology	Genetics
Hepatology	Infectious Disease	Microbiology
Nephrology	Neurology	Oncology
Pathology	Pediatrics	Rheumatology
Toxicology	Urology	Women's Health

HANA-HARMONIZED ASSIGNMENT OF NANOLITER ALIQUOTS®



17221: Bacterial 16s rDNA Sequencing

Print View

Components

NAME	METHOD	REFERENCE RANGE	UNITS
Specimen Source:			
Bacterial rDNA Sequencing			

Specimen Requirements

	TYPE	VOLUME	TEMPERATURE	INSTRUCTIONS
PRIMARY				
1	Pure Culture Agar Slant		Refrigerated - 14 Day(s)/Room Temperature - 14 Day(s)	

Clinical Utility

This test uses DNA sequencing and analysis of the 16S ribosomal RNA gene to identify bacteria at the genus and/or species level. Genotypic test are useful in the identification of unusual and new emerging pathogens. Genus and species information can be used to select appropriate therapeutic and management strategies.

Collection Instructions

Preferred specimen is culture isolate in an agar slant in a double
walled container. Mixed cultures will not be accepted. Subculture
isolate to the appropriate slant and submit in double walled
container.

Specimen Stability:
Room temperature and Refrigerated: Determined by Viability
Frozen: Unacceptable

Setup Schedule

Sunday, Tuesday, Thursday

Reported (Analytical Time)

9-13 days

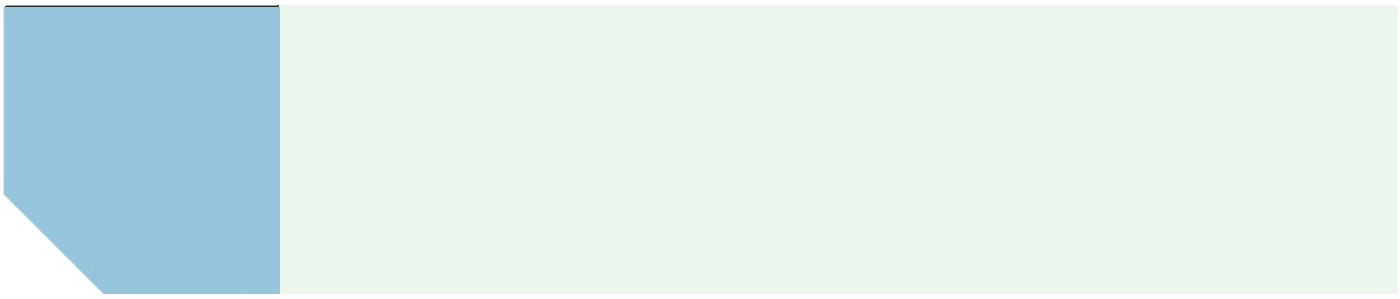
CPT Code

87153

The CPT codes provided are based on AMA guidelines and are for informational purposes only. CPT coding is the sole responsibility of the billing party. Please direct any questions regarding coding to the payer being billed.

Notes

Methodology: 16S Sequencing Reject Criteria: Mixed cultures or cultures shipped frozen Transport Temperature: Room temperature



Test Menu

16377: Clostridium Difficile Toxin B, Qualitative Real-Time PCR

Print View

Components

NAME	METHOD	REFERENCE RANGE	UNITS
C difficile, QL PCR	RT-PCR	Not Detected	

Specimen Requirements

	TYPE	VOLUME	TEMPERATURE	INSTRUCTIONS
PRIMARY				
1	Stool	5 (1) g	Frozen - 30 Day(s)/Refrigerated - 5 Day(s)	

Clinical Utility

Epidemiology studies demonstrate that an increased number of C. difficile-associated outbreaks have been reported worldwide, some with increased mortality and morbidity. This pathogen is the major cause of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis. The diagnosis of toxigenic C. difficile is usually done by tissue culture cytotoxicity assay and/or by C. difficile culture identification and/or by enzyme immunoassay (EIA). The tissue culture cytotoxicity assay and C. difficile culture identification are laborious and time consuming, and results are obtained within 3-5 days. Toxin EIA assays display low sensitivity. Molecular amplification techniques (PCR) allow the detection of only a few copies of bacterial DNA in clinical samples, better sensitivity and specificity. In addition, rapid PCR technology can achieve this in about 3 hours. The combination of these characteristics may allow the prompt targeted treatment of C. difficile-associated disease (CDAD) patients and thus a potential improved patient outcome and reduced recovery time.

Collection Instructions

Transfer liquid or soft stool (but not urine) into the container.
Avoid mixing toilet paper, or soap with the sample.
Store sample refrigerated until shipment.

Setup Schedule

Monday-Saturday

Reported (Analytical Time)

Same day

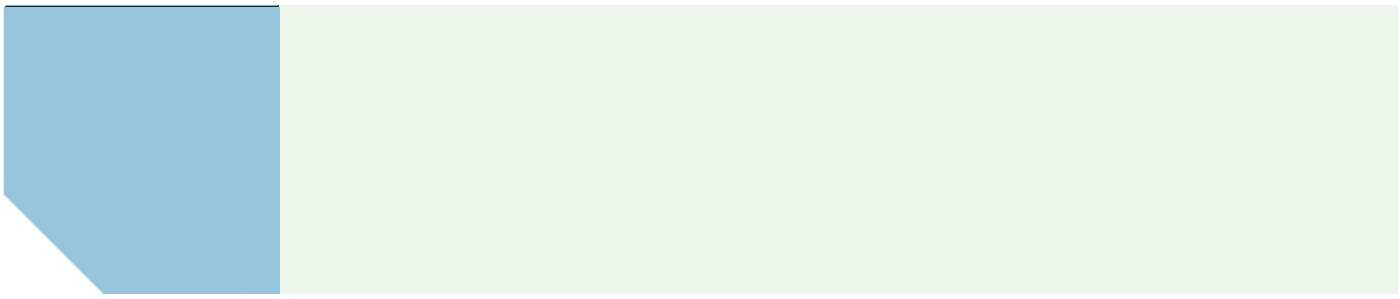
CPT Code

87493

The CPT codes provided are based on AMA guidelines and are for informational purposes only. CPT coding is the sole responsibility of the billing party. Please direct any questions regarding coding to the payer being billed.

Notes

Reject Criteria: Specimen other than liquid or semi-formed stool; Stool in preservative or mixed with urine; Specimen in wrong transport container



Test Menu

91427: Melanoma, Chromosomal Microarray, Clarisure® Oligo-SNP

Print View

Components

NAME	METHOD	REFERENCE RANGE	UNITS
Melanoma, ClariSure, Oligo			
Specimen Source:			
Diagnosis:			
Physician Name:			
Physician Phone #:			
Physician NPI			

Specimen Requirements

	TYPE	VOLUME	TEMPERATURE	INSTRUCTIONS
PRIMARY				
1	FFPE Tissue (Block/Slide)	1 (1) g	Refrigerated - 12 Month(s)/Room Temperature - 12 Month(s)	

Clinical Utility

This assay detects alterations in copy number or allelic imbalance. It can be used in the differential diagnosis of melanocytic lesions (e.g., classifying indeterminate lesions as benign or malignant). The Illumina Infinium oligo-SNP array reveals copy number variations and loss of heterozygosity (LOH) across the whole genome. The allelic copy number alterations, often complex, are associated with malignant melanoma. The assay is performed using DNA extracted from FFPE samples.

Collection Instructions

Formalin fixed paraffin-embedded block. Specimen must be fixed in 10%
neutral buffered formalin. Fixation between 6 and 48 hours is
recommended. Pathology report must accompany paraffin block.
Information required in this report include: Physician

identification, specimen identifiers (case and block number),
specimen site and type, tissue processing used (routine or
microwave), type of fixative, time and duration of fixation, and

Setup Schedule
Monday

Reported (Analytical Time)
21-22 days

CPT Code
81406+CM

The CPT codes provided are based on AMA guidelines and are for informational purposes only. CPT coding is the sole responsibility of the billing party. Please direct any questions regarding coding to the payer being billed.

Notes
METHODOLOGY:
Microarray SET UP:
Every other week

pathologic diagnosis.
Ship at room temperature. Do not freeze.

Exhibit 418

8

[Code of Federal Regulations]
[Title 21, Volume 8]
[Revised as of April 1, 2014]
[CITE: 21CFR864.4010]

TITLE 21--FOOD AND DRUGS
CHAPTER I--FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH AND HUMAN SERVICES
SUBCHAPTER H--MEDICAL DEVICES

PART 864 -- HEMATOLOGY AND PATHOLOGY DEVICES

Subpart E--Specimen Preparation Reagents

Sec. 864.4010 General purpose reagent.

(a) A general purpose reagent is a chemical reagent that has general laboratory application, that is used to collect, prepare, and examine specimens from the human body for diagnostic purposes, and that is not labeled or otherwise intended for a specific diagnostic application. It may be either an individual substance, or multiple substances reformulated, which, when combined with or used in conjunction with an appropriate analyte specific reagent (ASR) and other general purpose reagents, is part of a diagnostic test procedure or system constituting a finished in vitro diagnostic (IVD) test. General purpose reagents are appropriate for combining with one or more than one ASR in producing such systems and include labware or disposable constituents of tests; but they do not include laboratory machinery, automated or powered systems. General purpose reagents include cytological preservatives, decalcifying reagents, fixative and adhesives, tissue processing reagents, isotonic solutions and pH buffers. Reagents used in tests for more than one individual chemical substance or ligand are general purpose reagents (e.g., *Thermus aquaticus* (TAQ) polymerase, substrates for enzyme immunoassay (EIA)).

(b) *Classification.* Class I (general controls). The device is exempt from the premarket notification procedures in subpart E of part 807 of this chapter subject to the limitations in 864.9. If the device is not labeled or otherwise represented as sterile, it is exempt from the current good manufacturing practice requirements of the quality system regulation in part 820 of this chapter, with the exception of 820.180, with respect to general requirements concerning records, and 820.198, with respect to complaint files.

[45 FR 60592, Sept. 12, 1980, as amended at 54 FR 25045, June 12, 1989; 62 FR 62260, Nov. 21, 1997; 66 FR 38789, July 25, 2001]

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7. http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/Databases/ucm135680.htm
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Page Last Updated: 09/01/2014

Note: If you need help accessing information in different file formats, see Instructions for Downloading Viewers and Players.

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10903 New Hampshire Avenue
Silver Spring, MD 20993
Ph. 1-888-INFO-FDA (1-888-463-6332)
Email FDA

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Exhibit 419

Products and Medical Procedures

- In Vitro Diagnostics
- Proposed Pilot Triage Program
- Laboratory Developed Tests
- Companion Diagnostics
- Drugs of Abuse Tests
- Blood Glucose Monitoring Devices
- Home Use Tests
- Lab Tests

Laboratory Developed Tests

A laboratory developed test (LDT) is a type of in vitro diagnostic test that is designed, manufactured and used within a single laboratory.

LDTs can be used to measure or detect a wide variety of analytes (substances such as proteins, chemical compounds like glucose or cholesterol, or DNA), in a sample taken from a human body. Some LDTs are relatively simple tests that measure single analytes, such as a test that measures the level of sodium. Other LDTs are complex and measure or detect numerous analytes. For example, DNA variations can be detected from a blood sample, which can be used to help diagnose a genetic disease. Various levels of chemicals can be measured to help diagnose a patient's state of health, such as levels of cholesterol or sodium.

While the uses of an LDT are often the same as the uses of FDA-cleared or approved in vitro-diagnostic tests, some labs may choose to offer their own test. For example, a hospital lab may run its own vitamin D assay, even though there is an FDA-cleared test for vitamin D currently on the market.

The FDA does not consider diagnostic devices to be LDTs if they are designed or manufactured completely, or partly, outside of the laboratory that offers and uses them.

LDT's are important to the continued development of personalized medicine, but it is important that in vitro diagnostics are accurate so that patients and health care providers do not seek unnecessary treatments, delay needed treatments, or become exposed to inappropriate therapies.

The FDA has generally not enforced premarket review and other applicable FDA requirements because LDTs were relatively simple lab tests and generally available on a limited basis. But, due to advances in technology and business models, LDTs have evolved and proliferated significantly since the FDA first obtained comprehensive authority to regulate all in vitro diagnostics as devices in 1976. Some LDTs are now more complex, have a nation-wide reach and present higher risks, such as detection of risk for breast cancer and Alzheimer's disease, which are similar to those of other IVDs that have undergone premarket review.

The FDA has identified problems with several high-risk LDTs including: claims that are not adequately supported with evidence; lack of appropriate controls yielding erroneous results; and falsification of data. The FDA is concerned that people could initiate unnecessary treatment or delay or forego treatment altogether for a health condition, which could result in illness or death. The FDA is aware of faulty LDTs that could have led to: patients being over- or undertreated for heart disease; cancer patients being exposed to inappropriate therapies or not getting effective therapies; incorrect diagnosis of autism; unnecessary antibiotic treatments; and exposure to unnecessary, harmful treatments for certain diseases such as Lyme disease.

To help health care providers and patients better rely on the thousands of laboratory tests that are used every day to diagnose disease or other conditions or guide treatment and to encourage the advance of personalized medicine, on July 31, 2014 the FDA notified Congress of the Agency's intent to issue a draft oversight framework for LDTs based on risk to patients rather than whether they were made by a conventional manufacturer or a single laboratory. This draft oversight framework includes pre-market review for higher-risk LDTs, like those used to guide treatment decisions, including the many companion diagnostics that have entered the market as LDTs. In addition, under the draft framework, the FDA would continue to exercise enforcement discretion for low-risk LDTs and LDTs for rare diseases, among others. The framework would be phased in over many years.

Commenting on the Proposed LDT Regulatory Oversight Framework

As required by the 2012 FDA Safety and Innovation Act, on July 31, 2014 the FDA provided 60-day notice to Congress of its plan to issue draft guidance on the regulation of laboratory developed tests. At the same time, the FDA posted, for public viewing, the notification provided to Congress regarding LDTs that included the anticipated details of the draft guidances (**Notification to Congress: FDA's Laboratory Developed Tests Framework - July 31, 2014**).

Following this 60-day notice, on September 30, 2014, the FDA posted both draft guidances on its website. On October 3, 2014, the FDA published notices in the Federal Register formally announcing their release and the beginning of a 120-day public comment period. This comment period will last until February 2, 2015. Electronic comments on the draft LDT framework and notification guidances should be submitted through www.regulations.gov. Written comments may be submitted to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. Identify comments with the docket number found in brackets in the heading of the appropriate Federal Register notice for the draft guidance document (Docket No. FDA-2011-D-0360 for **Draft Guidance for Industry, Food and Drug Administration Staff, and Clinical Laboratories: Framework for Regulatory Oversight of Laboratory Developed Tests (LDTs)**, and Docket No. FDA-2011-D-0357 for **Draft Guidance for Industry, Food and Drug Administration Staff, and Clinical Laboratories: FDA Notification and Medical Device Reporting for Laboratory Developed Tests (LDTs)**).

The FDA welcomes comments on all aspects of these draft guidances, as well as on the following specific issues for the oversight framework draft guidance:

- **Traditional LDTs:** In Section D.5.(a) of the draft guidance, FDA has proposed continued enforcement discretion for premarket review and quality system requirements for a category of LDTs called "Traditional LDTs" based on whether the device is: 1) an LDT (designed, manufactured and used within a single laboratory); 2) manufactured and used by a health care facility laboratory (such as one located in a hospital or

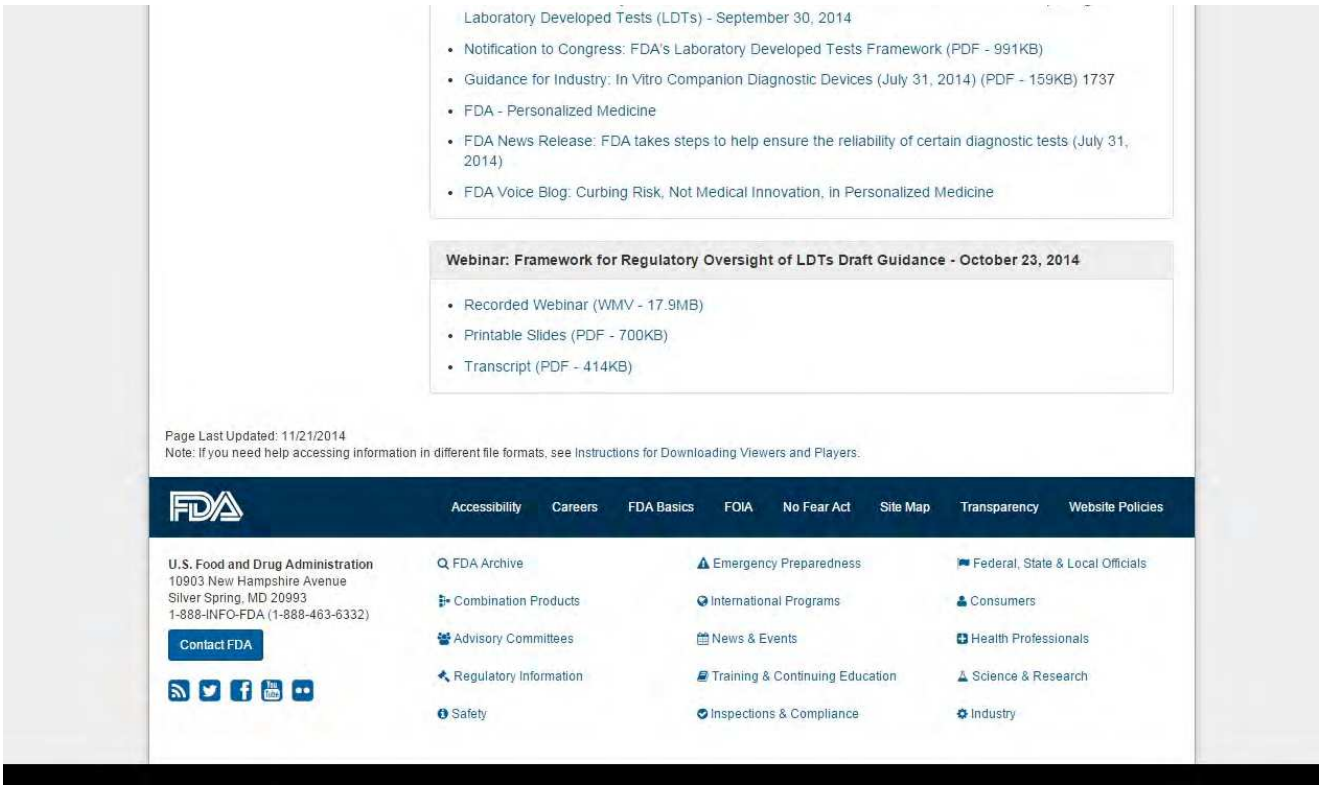
clinic) for a patient that is being diagnosed and/or treated at that same health care facility or within the facility's healthcare system; 3) comprised only of components and instruments that are legally marketed for clinical use; and 4) interpreted by qualified laboratory professionals without the use of automated instrumentation or software for interpretation. FDA believes that these factors appropriately mitigate risks associated with Traditional LDTs being used on patients so that continued enforcement discretion with respect to premarket review and quality system requirements is appropriate. However, FDA is seeking public feedback as to whether the following three factors may be sufficient to appropriately mitigate risk for this category of tests and whether they may also be sufficient to support continued enforcement discretion in full (i.e., for all regulatory requirements rather than just for premarket review and quality system requirements): 1) the test is an LDT (designed, manufactured and used within a single laboratory); 2) the test makes use of only components and instruments that are legally marketed for clinical use, which have a number of regulatory controls in place, including reporting of adverse events; and 3) the test is interpreted by laboratory professionals who are appropriately qualified and trained as required by the CLIA (Clinical Laboratory Improvement Amendments) regulations (*see, e.g.*, 42 CFR 493.1449), without the use of automated instrumentation or software for interpretation.

- **LDTs Used for Rare Diseases:** In Section D.5.(a) of the draft guidance, FDA has proposed continued enforcement discretion for premarket review and quality system requirements for LDTs used for rare diseases, which are those tests that meet the definition of LDT in the guidance (designed, manufactured and used within a single laboratory) and meet the definition of a Humanitarian Use Device (HUD) under 21 CFR 814.102(a)(5). With these factors, FDA has attempted to balance the need to mitigate the risks associated with these tests with their potential benefit for patients. FDA invites stakeholders to provide feedback on the suitability of these factors for LDTs for rare diseases. Further, FDA is seeking feedback on whether a factor other than the HUD definition should be considered, such as a factor based on the number of tests for a rare disease or condition that would likely (based on the prevalence of the condition) be conducted annually in the United States, and if so what the annual number of tests should be for the purpose of defining an LDT as an LDT for a rare disease. FDA also seeks feedback on whether enforcement discretion should be limited to tests that are designed, manufactured and used within a single laboratory.
- **Healthcare System:** In Section D.5. of the draft guidance, for the categories of tests called "Traditional LDTs" and "LDTs for Unmet Needs," FDA has identified factors it intends to consider in continuing to exercise enforcement discretion for premarket review and quality system requirements. One such factor is whether the LDT is both manufactured and used by a healthcare facility laboratory (such as one located in a hospital or clinic) for a patient that is being diagnosed and/or treated at that same health care facility or within that facility's healthcare system. To further clarify this factor, the guidance document explains that "healthcare system" refers to a collection of hospitals that are owned and operated by the same entity and that share access to patient care information for their patients, such as, but not limited to, drug order information, treatment and diagnosis information, and patient outcomes. While FDA invites feedback on all factors described in Section D.5. of the draft guidance, FDA specifically requests feedback on whether enforcement discretion should be limited, as proposed, to those LDTs that are both manufactured and used by a healthcare facility laboratory. FDA also invites the public to provide feedback to the Agency on which types of facilities would or would not be considered within a healthcare system, or to offer an alternative description of healthcare system for Agency consideration.
- **Quality System (QS) Phase-in:** In Section D.6. of the draft guidance, FDA has proposed to continue to exercise enforcement discretion with respect to QS regulation requirements, codified in 21 CFR Part 820, until a manufacturer of a given LDT submits a PMA or FDA issues a 510(k) clearance order for the LDT. Under this enforcement policy, the clinical laboratory manufacturing and using the LDT will be responsible for having a quality system in place that meets the minimum requirements codified in 21 CFR Part 820, either at the time of PMA submission (the facility that makes the device must pass an inspection as a condition of PMA approval as a matter of law (21 CFR 814.45(a)(3))), or prior to market launch for cleared devices, as applicable. FDA invites feedback on the timeframe for phase-in enforcement of QS regulation requirements. Specifically, FDA is considering whether those LDTs in the highest-risk category of devices (described in section D.5.(c)), which FDA intends to generally enforce premarket review requirements 12 months following publication of the final Framework guidance, should remain under enforcement discretion for the design control requirements (21 CFR 820.30(a-h) and (j)) of the QS regulation for up to 24 months after publication of the final guidance.
- **Notification:** FDA notes that some laboratory networks (i.e., more than one laboratory under the control of the same parent entity) offer the same test in multiple laboratories throughout their network. Although devices in this scenario do not meet FDA's definition of an LDT (i.e., they are not designed, manufactured and used within a single laboratory), FDA would like feedback on whether a single notification from the laboratory network for that test is sufficient, provided that the laboratory network indicates in the notification to FDA that the test is offered at multiple sites. In addition, FDA seeks comment on whether there are certain types of LDTs for which the Agency should neither enforce requirements for registration and listing nor request notification in lieu of registration and listing.
- FDA understands that members of the public may want more clarity around specific issues, such as how laboratory sponsors could interpret what elements make up a medical device, what might constitute the label or labeling for their device, whether or not unique device identifier requirements apply to LDTs, and how laboratory-physician communication about a test and its result would be viewed by FDA, among others. We invite public comment on these issues and any other issues or questions that should be addressed in the guidance, including how that issue or question should be addressed.

The agency also intends to hold a public meeting in early January 2015 to collect additional input during the comment period. When the FDA schedules the public meeting it will be announced in the Federal Register notice and on this website.

Additional Resources

- Public Workshop - Framework for Regulatory Oversight of Laboratory Developed Tests (LDTs), January 8-9, 2015
- Draft Guidance for Industry, Food and Drug Administration Staff, and Clinical Laboratories - Framework for Regulatory Oversight of Laboratory Developed Tests (LDTs) (PDF - 312KB)
  The comment period begins Oct. 3, 2014.
- Notification of Availability for the Draft Guidance Framework for Regulatory Oversight of Laboratory Developed Tests (LDTs) - September 30, 2014
- Draft Guidance for Industry, Food and Drug Administration Staff, and Clinical Laboratories - FDA Notification and Medical Device Reporting for Laboratory Developed Tests (LDTs) (PDF - 565KB)
  The comment period begins Oct. 3, 2014.
- Notification of Availability for the Draft Guidance FDA Notification and Medical Device Reporting for



Laboratory Developed Tests (LDTs) - September 30, 2014

- Notification to Congress: FDA's Laboratory Developed Tests Framework (PDF - 991KB)
- Guidance for Industry: In Vitro Companion Diagnostic Devices (July 31, 2014) (PDF - 159KB) 1737
- FDA - Personalized Medicine
- FDA News Release: FDA takes steps to help ensure the reliability of certain diagnostic tests (July 31, 2014)
- FDA Voice Blog: Curbing Risk, Not Medical Innovation, in Personalized Medicine

Webinar: Framework for Regulatory Oversight of LDTs Draft Guidance - October 23, 2014

- Recorded Webinar (WMV - 17.9MB)
- Printable Slides (PDF - 700KB)
- Transcript (PDF - 414KB)

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ILLUM-3950

Exhibit 420

Device Advice: Comprehensive Regulatory Assistance

- IVD Regulatory Assistance
- Clinical Laboratory Improvement Amendments (CLIA)
- CLIA Categorizations
- CLIA Waiver by Application
- Public Databases
- Overview of IVD Regulation

Overview of IVD Regulation

This section provides an overview of how FDA regulates IVDs. It does not operate to bind FDA or the Public. Manufacturers can find detailed information about complying with the Food, Drug and Cosmetic Act from the Device Advice: Device Regulation and Guidance section. For more information, see IVD Guidance Documents.

- What is an in vitro diagnostic product (IVD)?
- How are IVDs classified?
- What is a General Purpose Reagent?
- What are Analyte Specific Reagents?
- What are General Controls?
- What are the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88)?
- What is the "Pre-Submission" Process for IVDs?
- What is an Investigational Device Exemption (IDE)?
- What is a Premarket Notification [510(k)]?
- What is the 510(k) Paradigm?
- What is De Novo Classification for IVD devices?
- What is a Premarket Approval (PMA)?
- How does FDA regulate OIVD classified devices in clinical laboratory automation?
- What are the requirements for IVD labeling?
- How does FDA look at Quality Control?
- What is Establishment Registration?
- What is Medical Device Listing?
- What are Good Manufacturing Practices (GMPs) and Quality System Regulations (QSRs)?
- What is Medical Device Reporting?
- What happens to products that are in violation of laws administered by FDA?
- How does OIVD ensure compliance with the Federal Food, Drug, and Cosmetic Act?
- What is the Medical Device User Fee and Modernization Act of 2002 (MDUFMA)?

What is an in vitro diagnostic product (IVD)?

Definition: In vitro diagnostic products are those reagents, instruments, and systems intended for use in diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body. [21 CFR 809.3]

Regulatory Authority: IVDs are *medical devices* as defined in section 210(h) of the Federal Food, Drug, and Cosmetic Act, and may also be *biological products* subject to section 351 of the Public Health Service Act. Like other medical devices, IVDs are subject to premarket and postmarket controls. IVDs are also subject to the Clinical Laboratory Improvement Amendments (CLIA '88) of 1988.

Back to top

How are IVDs classified?

FDA classifies IVD products into Class I, II, or III according to the level of regulatory control that is necessary to assure safety and effectiveness. The classification of an IVD (or other medical device) determines the appropriate premarket process.

- Class I

- Class II
- Class III

The Code of Federal Regulations lists the classification of existing IVDs in 21 CFR 862, 21 CFR 864, and 21 CFR 866.

See also:

Device Advice: Classify Your Medical Device

Back to top

What is a General Purpose Reagent?

A general purpose reagent (GPR) is "a chemical reagent that has general laboratory application, is used to collect, prepare, and examine specimens from the human body for diagnostic purposes, and is not labeled or otherwise intended for a specific diagnostic application ...[General purpose reagents] do not include laboratory machinery, automated or powered systems."

Classification information for GPRs can be found in 21 CFR 864.4010(a).

Back to top

What are Analyte Specific Reagents?

Analyte specific reagents (ASRs) are "antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens."

Classification information for ASRs can be found in 21 CFR 864. 4020(a).

Back to top

What are General Controls?

IVDs, and all other medical devices, are subject to General Controls.

General Controls are the basic provisions (authorities) of the May 28, 1976 Medical Device Amendments to the Food, Drug and Cosmetic Act, that provide the FDA with the means of regulating devices to ensure their safety and effectiveness. The General Controls in the Amendments apply to all medical devices, including IVDs. They include provisions that relate to adulteration; misbranding; device registration and listing; premarket notification; banned devices; notification, including repair, replacement, or refund; records and reports; restricted devices; and good manufacturing practices.

See also:

Device Advice: General Controls for Medical Devices

Back to top

What are the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88)?

- CLIA '88 establishes quality standards for laboratory testing and an accreditation program for clinical laboratories.
- CLIA '88 requirements vary according to the technical complexity in the testing process and risk of harm in reporting erroneous results. The regulations established three categories of testing on the basis of the complexity of the testing methodology: a) waived tests, b) tests of moderate complexity, and c) tests of high complexity.
- Manufacturers apply for CLIA '88 categorization during the premarket process.
- Under CLIA, laboratories performing only waived tests are subject to minimal regulation. Laboratories performing moderate or high complexity tests are subject to specific laboratory standards governing certification, personnel, proficiency testing, patient test management, quality assurance, quality control, and inspections.

See also:

CLIA '88 - Clinical Laboratory Improvement Amendments Home Page

Search CLIA '88 Database

Back to top

What is the "Pre-Submission" Process for IVDs?

The Pre-Submission" process was established under MDUFA III and defined in the Commitment Letter as:

> A Pre-Submission includes a formal written request from an applicant for feedback from the FDA which is provided in the form of a formal written response or, if the manufacture chooses, a meeting or teleconference in which the feedback is documented in meeting minutes. A Pre-Submission meeting is a meeting or teleconference in which the FDA provides its substantive feedback on the Pre-Submission.
>
> A Pre-Submission provides the opportunity for an applicant to obtain FDA's feedback prior to submission of an Investigational Device Exemption (IDE) or marketing application. The request must include specific questions regarding review issues relevant to a planned IDE or marketing application (e.g., questions regarding pre-clinical and clinical testing protocols or data requirements). A Pre-Submission is appropriate when FDA's feedback on specific questions is necessary to guide product development and/or application preparation.

The FDA encourages use of the Pre-Submission program under circumstances such as the following:

- The device involves new technology, a new intended use, or a new analyte and it will be helpful to familiarize the FDA with the novel features in advance of the submission;
- Assistance is needed in defining possible regulatory pathways;
- The studies involve complex data and/or statistical approaches;
- The predicate or reference method is unclear or uncertain; or
- The new device is a multiplex device capable of simultaneously testing a large number of analytes.

A sponsor should submit a Pre-Submission if they would like the FDA's thoughts on their studies or proposals *prior* to starting their studies. The potential benefits of submitting a Pre-Submission are:

- to begin a dialogue with FDA and promote greater understanding.
- to reduce the cost of research studies by focusing in on the important information needed for FDA approval (or clearance) and eliminating unnecessary or burdensome studies, and
- to speed the review process for the future marketing application since FDA will already be familiar with the device.

Pre-Submissions and meetings are strictly *voluntary*, and any comments or recommendations made in the review of protocols or during these meetings are *not binding* on the manufacturer or the Agency.

See also:

> Device Advice: Pre-Submission Process
>
> Requests for Feedback on Medical Device Submissions: The Pre-Submission Program and Meetings with Food and Drug Administration Staff

Back to top

What is an Investigational Device Exemption (IDE)?

- An IDE allows an investigational device to be used in a clinical study in order to collect safety and effectiveness data to support PMA or 510(k) submission.
- An IDE permits devices to be shipped lawfully for the purpose of conducting investigations without complying with requirements of the FD&C Act that apply to devices in commercial distribution.
- Many IVDs are exempt from IDE requirements.

See also:

> Device Advice: Clinical Trials and Investigational Device Exemption
>
> Guidance for FDA Staff: Regulating In Vitro Diagnostic Device (IVD) Studies

Back to top

What is a Premarket Notification [510(k)]?

- A 510(k) is a premarketing submission made to FDA to demonstrate that the device to be marketed is as safe and effective, that is, substantially equivalent (SE), to a legally marketed device that is not subject to premarket approval (PMA).
- Each person who wants to market Class I, II and some III devices intended for human use in the U.S. must submit a 510(k) to FDA at least 90 days before marketing unless the device is exempt from 510(k) requirements.
- FDA reviews 510(k) submissions in a 90-day timeline. If there are unaddressed scientific issues, the review

scientists can ask for additional information and put the submission temporarily on hold.

- If FDA finds the information provided by the sponsor meets the standard of equivalency, the product is cleared for marketing in the United States. If FDA finds that there is no predicate for the device, or that the new device does not have equivalent performance to the identified predicate, then the device is found not substantially equivalent.
- There is no 510(k) form but instead a format for the submission is described in 21 CFR 807.

Back to top

510(k) Review

Review of a 510(k) is based on the evaluation of the analytical performance characteristics of the new device compared to the predicate, including:

- the bias or inaccuracy of the new device;
- the imprecision of the new device; and
- the analytical specificity and sensitivity.

Studies Required to Demonstrate Substantial Equivalence

The types of studies required to demonstrate substantial equivalence include the following:

- In the majority of cases, analytical studies using clinical samples (sometimes supplemented by carefully selected artificial samples) will suffice.
- For some IVDs, the link between analytical performance and clinical performance is not well defined. In these circumstances, clinical information may be required.
- FDA rarely requires prospective clinical studies for IVDs, but regularly requests clinical samples with sufficient laboratory and/or clinical characterization to allow an assessment of the clinical validity of a new device. This is usually expressed in terms of clinical sensitivity and clinical specificity or agreement.

Limitations to FDA Review

There are several limitations to FDA's review of 510(k) applications:

- FDA does not have the facilities to perform wet lab product evaluation, instead it bases its review on the materials submitted by the sponsor;
- There are few performance standards on which to ensure that regulatory decisions are based on clearly defined scientific parameters.

See also:

Device Advice: Premarket Notification [510(k)]

In Vitro Diagnostic Devices: Guidance for the Preparation of 510(k) Submissions

Back to top

What is the 510(k) Paradigm?

- The 510(k) Paradigm presents device manufacturers with two new optional approaches for obtaining marketing clearance for devices subject to 510(k) requirements:
  - Special 510(k) Option for manufacturers who modify their own legally-marketed device
  - Abbreviated 510(k) Option when a guidance document exists, a Special Control has been established, or FDA has recognized a relevant consensus standard.
- A manufacturer considering either a special or abbreviated 510(k) should first consult 21 CFR 807.81(a)(3).

See also:

The New 510(k) Paradigm: Alternate Approaches to Demonstrating Substantial Equivalence in Premarket Notifications.

Guidance for Industry: Frequently Asked Questions on the New 510(K) Paradigm

Back to top

What is De Novo Classification for IVD Devices?

Prior to the FDA Modernization Act of 1997 (FDAMA), all devices on the market as of May 28, 1976 were classified according to their risk. Any device that was not classified was automatically assigned to Class III, requiring a premarket approval (PMA) application. A device could be moved out of Class III only through a cumbersome reclassification process.

FDAMA amended Section 513(f) to provide a new mechanism for classifying new Class III devices for which there is no predicate device. It allows the recipient of an NSE (not substantially equivalent) letter to request a risk-based classification determination to be made for the device.

In some cases, this allows a manufacturer to use the De Novo process to submit a 510(k) for a new IVD that would otherwise have to get to market via the PMA process.

See also:

FDA Modernization Act of 1997: Evaluation of Automatic Class III Designation

New Section 513(f)(2) - Evaluation of Automatic Class III Designation, Guidance for Industry and CDRH Staff

Back to top

What is a Premarket Approval (PMA)?

- A PMA is an application submitted to FDA to request approval to market, or continue marketing, a class III medical device.
- PMA approval is based on scientific evidence providing a reasonable assurance that the device is safe and effective for its intended use or uses. For IVDs, there is a unique link between safety and effectiveness since the safety of the device is not generally related to contact between the device and patient. For IVD products, the safety of the device relates to the impact of the device's performance, and in particular on the impact of false negative and false positive results, on patient health.
- FDA reviews PMA submissions in a 180-day timeline. If there are unaddressed scientific issues, the review scientists can ask for additional information and put the submission temporarily on hold. If a product is a first of a kind, or if it presents unusual issues of safety and effectiveness, it is generally reviewed before it is approved by an advisory panel of outside experts. Approval of a PMA requires review of the manufacturing processes, an inspection of the manufacturing facility, a bioresearch monitoring audit of clinical data sites, as well as comprehensive review of the premarket data.
- If FDA finds that a product is safe and effective, it receives an official approval order for marketing in the United States. If FDA finds that a product is not safe and effective, it may be non-approved.
- A manufacturer considering a PMA should consult 21 CFR 814.

Studies Required to Demonstrate Safety and Effectiveness

For most PMAs, sponsors identify surrogate endpoints and establish the device performance (clinical sensitivity and specificity or agreement) with relation to the identified endpoints in corollary studies using randomly collected clinical studies.

Limitations to FDA Review

There are several limitations to FDA's review of PMA applications:

- Lack of a "gold standard" against which to judge performance;
- Bias may occur in the collection of data to establish safety and effectiveness, through problems in the study design or conduct;
- It can be challenging to determine the minimum performance required for approval.

See also:

Device Advice: Premarket Approval

Back to top

How does FDA regulate OIVD classified devices in clinical laboratory automation?

Definitions:*

***automated instrument**– a laboratory instrument that may or may not be connected to a laboratory information system (LIS), hospital information system (HIS), and/or laboratory automation system (LAS), which performs measurements on a patient's sample; **NOTE:** These instruments may have specific hardware and/or software modifications that allow interface to a laboratory automation system.*

***laboratory automation system, LAS** – a system of information and hardware technology that allows the operation of the clinical laboratory process without significant operator intervention; **NOTE:** Typical functionality includes information system control of the instruments through direct LAS interfacing, including any technology that manipulates the specimen (i.e., centrifuge); transportation of the specimen; result evaluation, repeat testing, reflex testing; and quality assessment and results reporting.*

***laboratory information system, LIS** - the information system that is responsible for management of data regarding patient specimen identification, tests requested, results reported, quality control testing, and other*

*regarding patient specimen identification, tests requested, results reported, quality control testing, and other aspects of sample analysis; **NOTES:** a) The LIS interfaces directly with the LAS to communicate patient, visit, container, test orders, specimen status, and results about specific testing to be done; b) Instrument or specimen processing and handling devices may be interfaced with the LIS or the LAS to direct specific testing and to retrieve results for reporting; c) The LIS is frequently also interfaced to a clinical information system for use by physicians and other medical personnel.*

Clinical laboratory automation helps diagnose health conditions in a timely and effective manner. OIVD uses a risk-based regulatory approach to these products to ease the burden on industry while ensuring public safety.

Stand alone automated clinical analyzers are exempt class I devices and do not require Premarket Notification [510(k)].

When an automated clinical analyzer measures a specific analyte, the analyzer plus the associated reagents become a test system. These test systems are considered Combination Devices, i.e., devices combined with devices, and are NOT considered Combination Products, and are classified in the highest of the predicate device classifications. Therefore automated clinical analyzers are not exempt from premarket notification when they include any class I reserved device or a class II device. Automated clinical analyzers that include class III devices are subject to Premarket Approval.

Laboratory Information Systems (LIS) and Laboratory Automation Systems (LAS) meet the definition of combination devices when a manufacturer makes an integration claim to a specified analyzer, with the following exceptions:

If the device only receives information for the patient record and neither transmits any instructions to any interfaced device (other than test orders) nor controls any device functions or alarms, no premarket notification is required.

If the device performs only simple physiological and clinical calculations, as long as the algorithms are established and well accepted by the clinical community, no premarket notification is required.

Examples of when the LAS takes over the function of an analyzer:

If the LAS takes over the pipetting that the analyzer normally does for analysis, or the LAS hands off the sample ID directly to the analyzer without the analyzer performing the sample identification, then a premarket notification IS required.

Regardless of classification, devices automated with computer software are subject to Design Controls under the Quality System Regulation 21 CFR Part 820.

*Clinical and Laboratory Standards Institute. Laboratory Automation: Bar Codes for Specimen Container Identification; Approved Standard - Second Edition. CLSI document AUTO2-A2 [ISBN 1-56238-000-0]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2005.

Back to top

What are the Requirements for IVD Labeling?

In Vitro Diagnostic Products have special labeling requirements under 21 CFR 809, Subpart B, In Vitro Diagnostic Products for Human Use. Before a manufacturer obtains clearance or approval for an IVD product, they must label the product in accordance with labeling regulations.

See also:

Device Advice: Labeling Requirements for In Vitro Diagnostic Devices

Points to Consider Regarding Labeling and Premarket Submissions for Home Use In Vitro Diagnostic Devices

Back to top

How does FDA Look at Quality Control?

- Quality control (QC) is a material or mechanism which, when used with or as part of a test system, monitors the analytical performance of that test system. It may monitor the entire test system or only one aspect of it.
- FDA regulates the *material or mechanism* **as a medical device**; it does not monitor how a QC component is used within a laboratory (i.e. how often other QC types should be run, or whether the QC replaces other, more traditional external types of QC).
- FDA makes sure that products have QC materials, reviews labeling for accuracy, and determines if manufacturers have protocols to ensure stability.
- How a QC component is used within a laboratory is not within FDA's jurisdiction. Other Agencies, such as of the Centers for Medicare and Medicaid Services (CMS), the College of American Pathologists (CAP), or the Joint Commission Accrediting Hospital Organization (JCAHO) have jurisdiction over the procedures and practices within laboratories.

- A manufacturer developing a quality control material or mechanism should consult 21 CFR 862.1660 and 21 CFR 862.9.

See also:

Internal Quality Control Testing; Principles and Definitions; NCCLS C24-A

Back to top

What is Establishment Registration?

- Establishments involved in the production and distribution of medical devices intended for commercial distribution in the United States (U.S.) are required to register with the FDA.
- Registration provides FDA with the location of medical device manufacturing facilities and importers.
- Registration of an establishment is not an approval of the establishment or its devices by FDA. That is, it does not provide FDA clearance to market. Unless exempt, premarketing clearance is required before a device can be placed into commercial distribution in the U.S.

See also:

Device Advice: Establishment Registration

Back to top

What is Medical Device Listing?

- Most medical device establishments required to register with FDA must list the devices they have in commercial distribution including devices produced exclusively for export.
- Listing keeps FDA advised of the generic category(s) of devices an establishment is marketing.
- Listing of a device does not provide FDA clearance to market. Unless exempt, premarketing clearance is required before a device can be placed into commercial distribution in the U.S.

See also:

Device Advice: Medical Device Listing

Back to top

What are Good Manufacturing Practices (GMPs) and Quality System Regulations (QSRs)?

The GMP requirements are part of the Quality System Regulations. They require that domestic or foreign manufacturers have a quality system for the design, manufacture, packaging, labeling, storage, installation, and servicing of finished medical devices intended for commercial distribution in the United States. The QS Regulation is contained in 21 CFR 820.

See also:

Device Advice: Good Manufacturing Practices (GMP) / Quality System (QS) Regulation

Back to top

What is Medical Device Reporting?

- The Medical Device Reporting (MDR) regulations require manufacturers who have received complaints of device malfunctions, serious injuries or deaths associated with medical devices to notify FDA of the incident.
- MDR regulations require User Facilities (e.g., hospitals, laboratories) to report suspected medical device related deaths to both the FDA and the manufacturers. User facilities must report medical device related serious injuries to the manufacturer.

See also:

Medical Device Reporting (MDR)

Device Advice: Medical Device Reporting (MDR)

Back to top

What happens to products that are in violation of laws administered by FDA?

- In most cases, manufacturers and distributors voluntarily recall products that present a risk of injury or gross deception or are otherwise defective. 21 CFR 7 provides guidance so that responsible firms may conduct an effective recall.
- In rare instances, where the manufacturer or importer fails to voluntarily recall a device that is a risk to health, FDA may issue a recall order to the manufacturer under 21 CFR 810, Medical Device Recall Authority.
- Under 21 CFR 806, Medical Device Correction and Removals, manufacturers (including refurbishers and reconditioners) and importers are required to make a report to FDA of any correction or removal of a medical device(s) if the correction or removal was initiated to reduce a risk to health posed by the device or to remedy a violation of the Act caused by the device which may present a risk to health.

See also:

Device Advice: Medical Device Recalls and Corrections and Removals

Back to top

How does OIVD ensure compliance with the Federal Food, Drug, and Cosmetic Act?

OIVD works with manufacturers throughout the total product life cycle to ensure compliance with the Medical Device Amendments in the least burdensome manner. This approach enables manufacturers to receive assistance and feedback in a timely manner and may reduce the need for compliance actions.

Back to top

What is the Medical Device User Fee and Modernization Act of 2002 (MDUFMA)?

The Medical Device User Fee and Modernization Act of 2002 (MDUFMA), P.L. 107-250, amends the Federal Food, Drug, and Cosmetic Act to provide FDA important new responsibilities, resources, and challenges. MDUFMA was signed into law October 26, 2002. MDUFMA has three particularly significant provisions:

- It establishes fees for premarket reviews of medical devices;
- It permits establishment inspections by accredited persons (third-parties); and
- It provides new regulatory requirements for reprocessed single-use devices.

See also:

Medical Device User Fee and Modernization Act (MDUFMA) of 2002

Back to top

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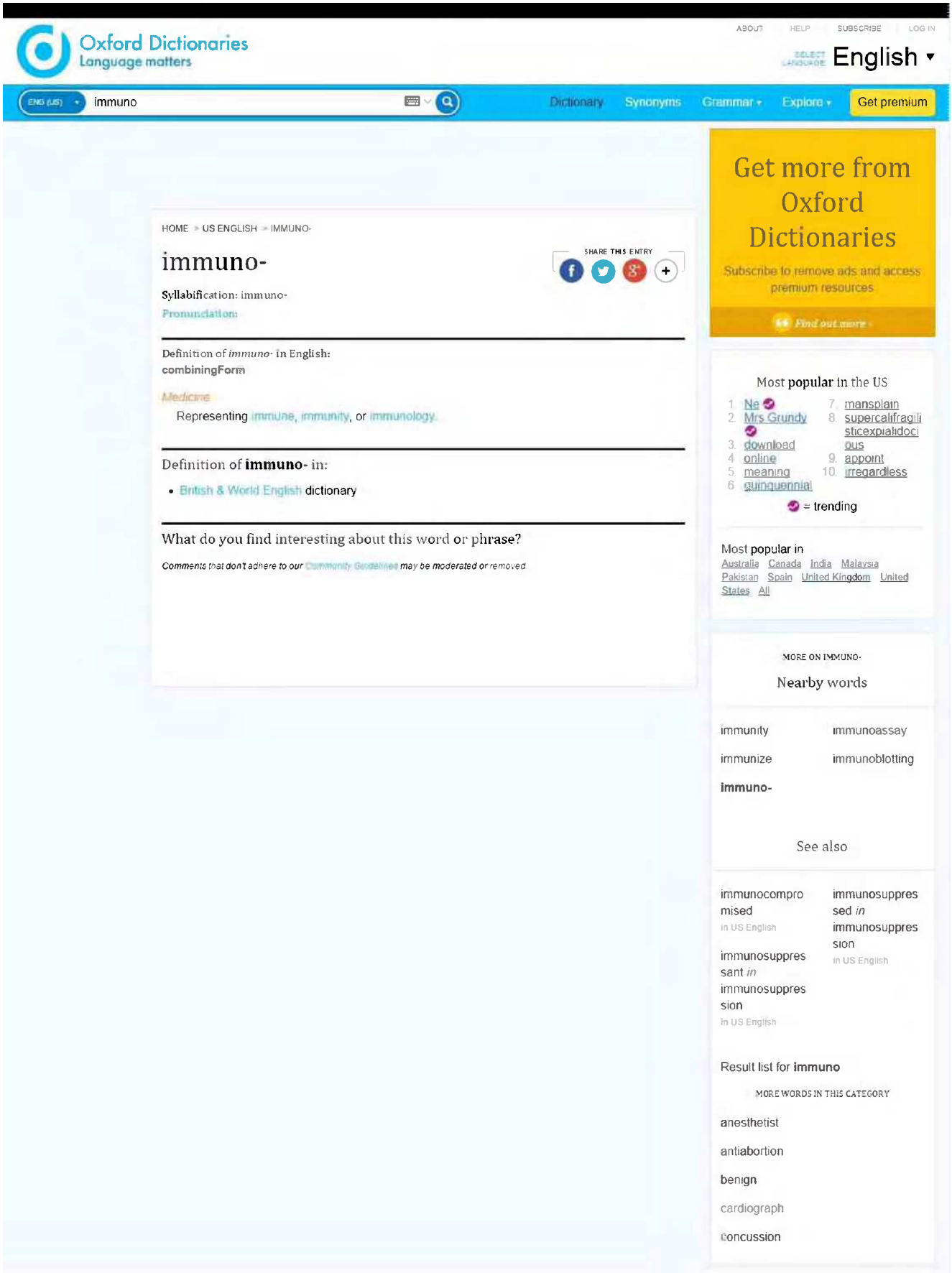
Exhibit 421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immuno-
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Syllabification: immuno-
Pronunciation:
Definition of immuno- in English:
combiningForm
Medicine
Representing immune, immunity, or immunology.
Definition of immuno- in:
British & World English dictionary
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Nearby words
immunity
immunize
immuno-
immunoassay
immunoblotting
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immunocompromised
in US English
immunosuppressed in immunosuppression
in US English
immunosuppressant in immunosuppression
in US English
Result list for immuno
MORE WORDS IN THIS CATEGORY
anesthetist
antiabortion
benign
cardiograph
concussion

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WORD OF THE DAY
prevaricate
Pronunciation pri vere kāt
VERB
speak or act in an evasive way
See full definition ›
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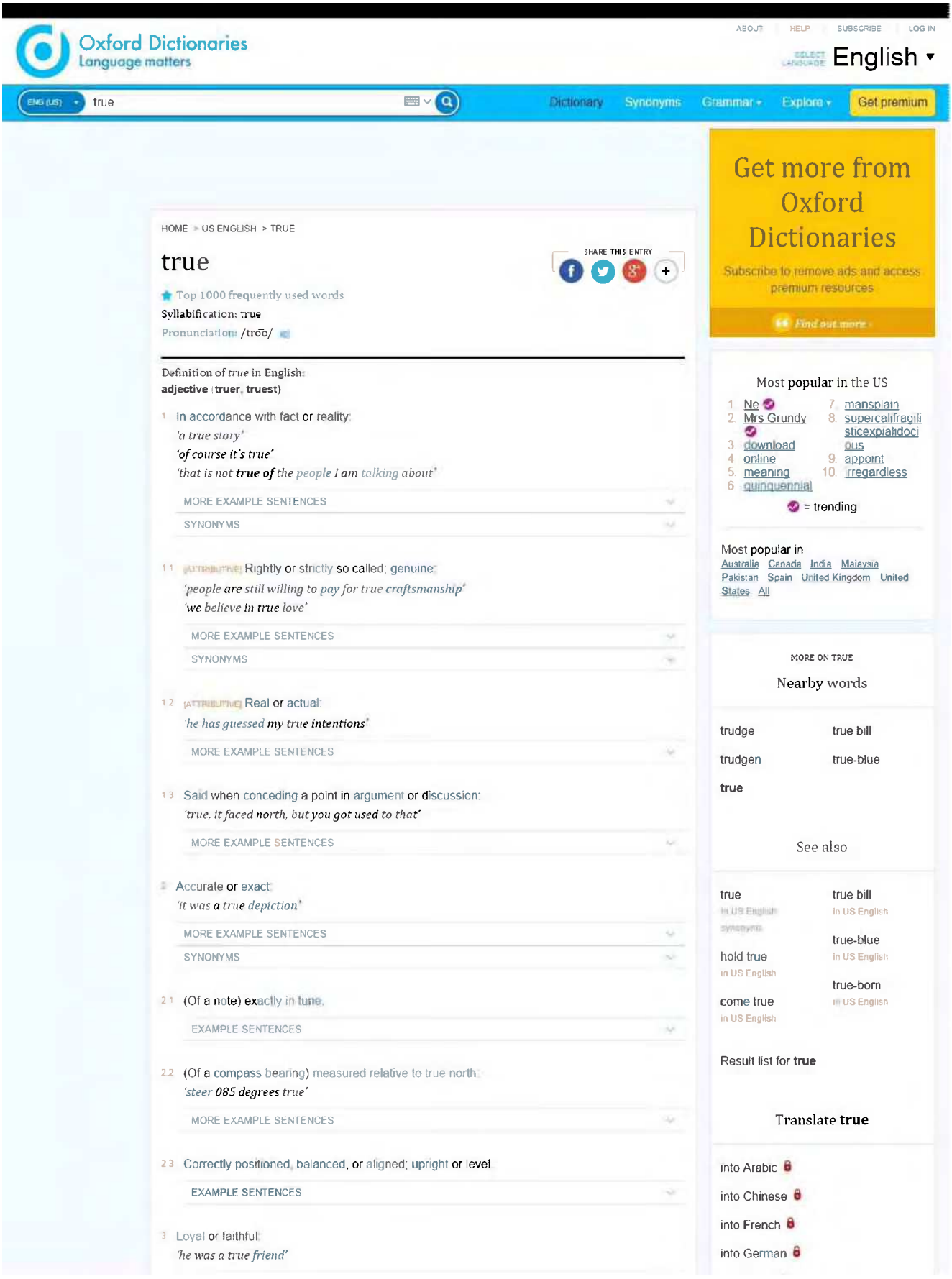

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ENG (US) true

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HOME > US ENGLISH > TRUE

true

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Top 1000 frequently used words
Syllabification: true
Pronunciation: /trōō/

Definition of *true* in English:
adjective (**truer**, **truest**)

1 In accordance with fact or reality:
'a true story'
'of course it's true'
*'that is not **true of** the people I am talking about'*

MORE EXAMPLE SENTENCES
SYNONYMS

1.1 (ATTRIBUTIVE) Rightly or strictly so called; genuine:
'people are still willing to pay for true craftsmanship'
'we believe in true love'

MORE EXAMPLE SENTENCES
SYNONYMS

1.2 (ATTRIBUTIVE) Real or actual:
'he has guessed my true intentions'

MORE EXAMPLE SENTENCES

1.3 Said when conceding a point in argument or discussion:
'true, it faced north, but you got used to that'

MORE EXAMPLE SENTENCES

2 Accurate or exact:
'it was a true depiction'

MORE EXAMPLE SENTENCES
SYNONYMS

2.1 (Of a note) exactly in tune.

EXAMPLE SENTENCES

2.2 (Of a compass bearing) measured relative to true north:
'steer 085 degrees true'

MORE EXAMPLE SENTENCES

2.3 Correctly positioned, balanced, or aligned; upright or level.

EXAMPLE SENTENCES

3 Loyal or faithful:
'he was a true friend'

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MORE ON TRUE

Nearby words

trudge
trudgen
true
true bill
true-blue

See also

true
in US English
synonyms
hold true
in US English
come true
in US English
true bill
in US English
true-blue
in US English
true-born
in US English

Result list for **true**

Translate **true**

into Arabic
into Chinese
into French
into German

ILLUM-3961

MORE EXAMPLE SENTENCES

SYNONYMS

3.1 [PREDICATIVE] (**true to**) Accurately conforming to (a standard or expectation); faithful to:
'this entirely new production remains true to the essence of Lorca's play'

MORE EXAMPLE SENTENCES

4 chiefly *archaic* Honest:
'we appeal to all good men and true to rally to us'

MORE EXAMPLE SENTENCES

adverb Back to top

1 chiefly *literary* Truly:
'Hobson spoke truer than he knew'

MORE EXAMPLE SENTENCES

2 Accurately or without variation:

EXAMPLE SENTENCES

verb (**trues**, **truing** or **trueing**, **trued**) Back to top

[WITH OBJECT]
Bring (an object, wheel, or other construction) into the exact shape, alignment, or position required.

EXAMPLE SENTENCES

Origin

Old English *trēowe*, *trȳwe* 'steadfast, loyal'; related to Dutch *getrouw*, German *treu*, also to truce.

MORE

Phrases

come true
1 Actually happen or become the case:
'dreams can come true'

MORE EXAMPLE SENTENCES

out of true
2 Not in the correct or exact shape or alignment:
'take care not to pull the frame out of true'

MORE EXAMPLE SENTENCES

many a true word is spoken in jest
3 *proverb* A humorous remark not intended to be taken seriously may turn out to be accurate after all.

EXAMPLE SENTENCES

true to form (or **type**)
4 Being or behaving as expected:
'true to form, they took it well'

MORE EXAMPLE SENTENCES

true to life
5 Accurately representing real events or objects:
'artworks of the period were often composed in strident colors not true to life'

MORE EXAMPLE SENTENCES

ILLUM-3962

Derivatives

trueness

Pronunciation: /'trōōnəs/

noun

EXAMPLE SENTENCES

Words that rhyme with true

accrue, adieu, ado, anew, Anjou, aperçu, askew, ballyhoo, bamboo, bedew, bestrew, billet-doux, blew, blue, boo, boohoo, brew, buckaroo, canoe, chew, clew, clou, clue, cock-a-doodle-doo, cockatoo, construe, coo, Corfu, coup, crew, Crewe, cru, cue, déjà vu, derring-do, dew, didgeridoo, do, drew, due, endue, ensue, eschew, feu, few, flew, flu, flue, foreknew, glue, gnu, goo, grew, halloo, hereto, hew, Hindu, hitherto, how-do-you-do, hue, Hugh, hullabaloo, imbrue, imbue, jackaroo, Jew, kangaroo, Karroo, Kathmandu, kazoo, Kiangsu, knew, Kru, K2, kung fu, Lahu, Lanzhou, Lao-tzu, lasso, lieu, loo, Lou, Manchu, mangetout, mew, misconstrue, miscue, moo, moue, mu, nardoo, new, non-U, nu, ooh, outdo, outflew, outgrew, peekaboo, Peru, pew, plew, Poitou, pooh, pooh-pooh, potoroo, pursue, queue, revue, roo, roux, rue, Selous, set-to, shampoo, shih-tzu, shoe, shoo, shrew, Sioux, skean dhu, skew, skidoo, slew, smew, snafu, sou, spew, sprue, stew, strew, subdue, sue, switcheroo, taboo, tattoo, thereto, thew, threw, thro, through, thru, tickety-boo, Timbuktu, tiramisu, to, to-do, too, toodle-oo, true-blue, tu-whit tu-whoo, two, vendue, view, vindaloo, virtu, wahoo, wallaroo, Waterloo, well-to-do, whereto, whew, who, withdrew, woo, Wu, yew, you, zoo

Definition of **true** in:

- British & World English dictionary
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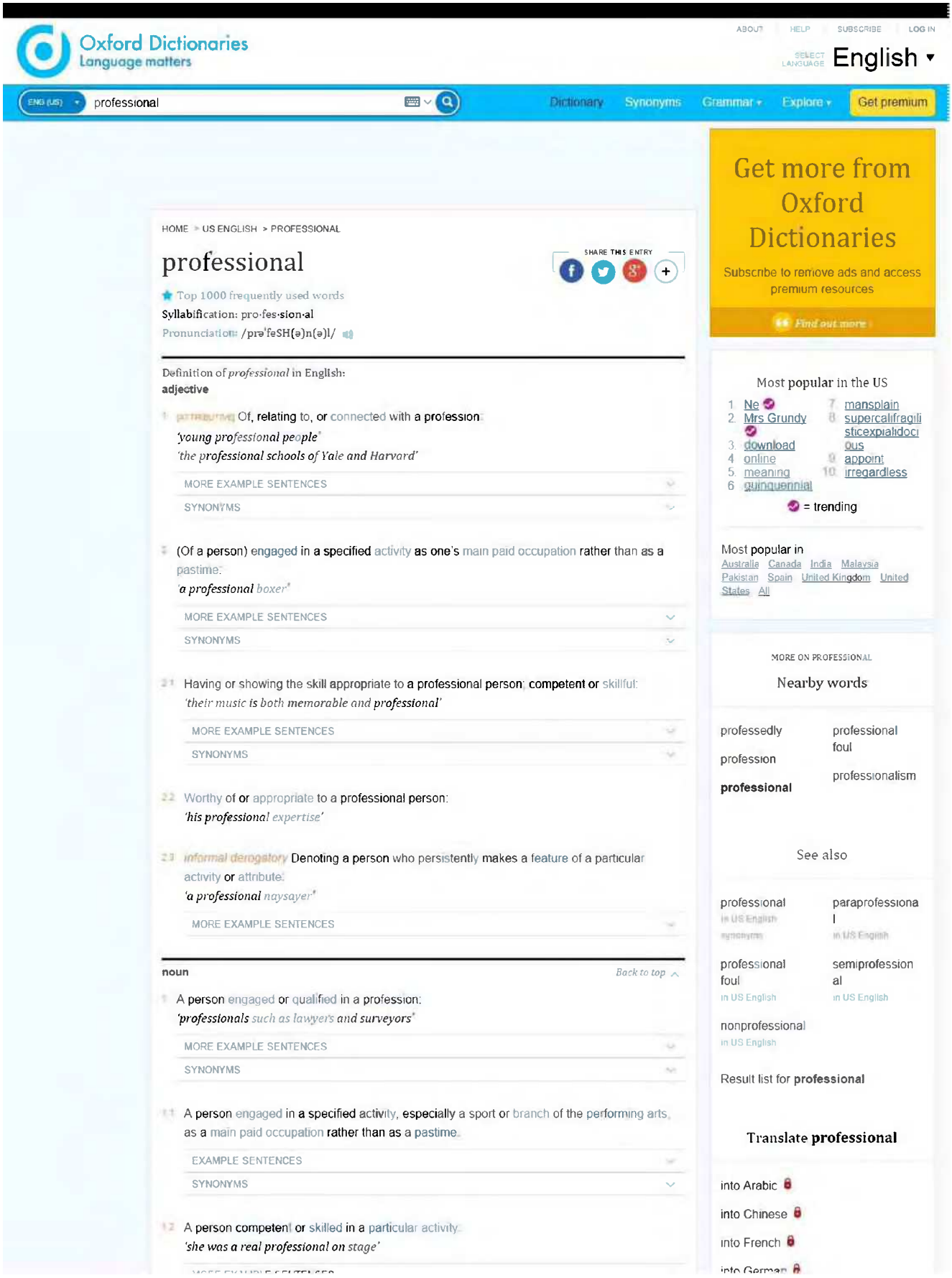

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professional

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Top 1000 frequently used words
Syllabification: pro·fes·sion·al
Pronunciation: /prəˈfeSH(ə)n(ə)l/

Definition of *professional* in English:
adjective

1 ATTRIBUTIVE Of, relating to, or connected with a profession:
'young professional people'
'the professional schools of Yale and Harvard'
MORE EXAMPLE SENTENCES
SYNONYMS

2 (Of a person) engaged in a specified activity as one's main paid occupation rather than as a pastime:
'a professional boxer'
MORE EXAMPLE SENTENCES
SYNONYMS

2.1 Having or showing the skill appropriate to a professional person; competent or skillful:
'their music is both memorable and professional'
MORE EXAMPLE SENTENCES
SYNONYMS

2.2 Worthy of or appropriate to a professional person:
'his professional expertise'

2.3 *informal derogatory* Denoting a person who persistently makes a feature of a particular activity or attribute:
'a professional naysayer'
MORE EXAMPLE SENTENCES

noun Back to top

1 A person engaged or qualified in a profession:
'professionals such as lawyers and surveyors'
MORE EXAMPLE SENTENCES
SYNONYMS

1.1 A person engaged in a specified activity, especially a sport or branch of the performing arts, as a main paid occupation rather than as a pastime:
EXAMPLE SENTENCES
SYNONYMS

1.2 A person competent or skilled in a particular activity:
'she was a real professional on stage'

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MORE ON PROFESSIONAL

Nearby words
professedly
profession
professional
professional foul
professionalism

See also
professional in US English synonyms
paraprofessional in US English
professional foul in US English
semiprofessional in US English
nonprofessional in US English

Result list for **professional**

Translate **professional**
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into Chinese
into French
into German

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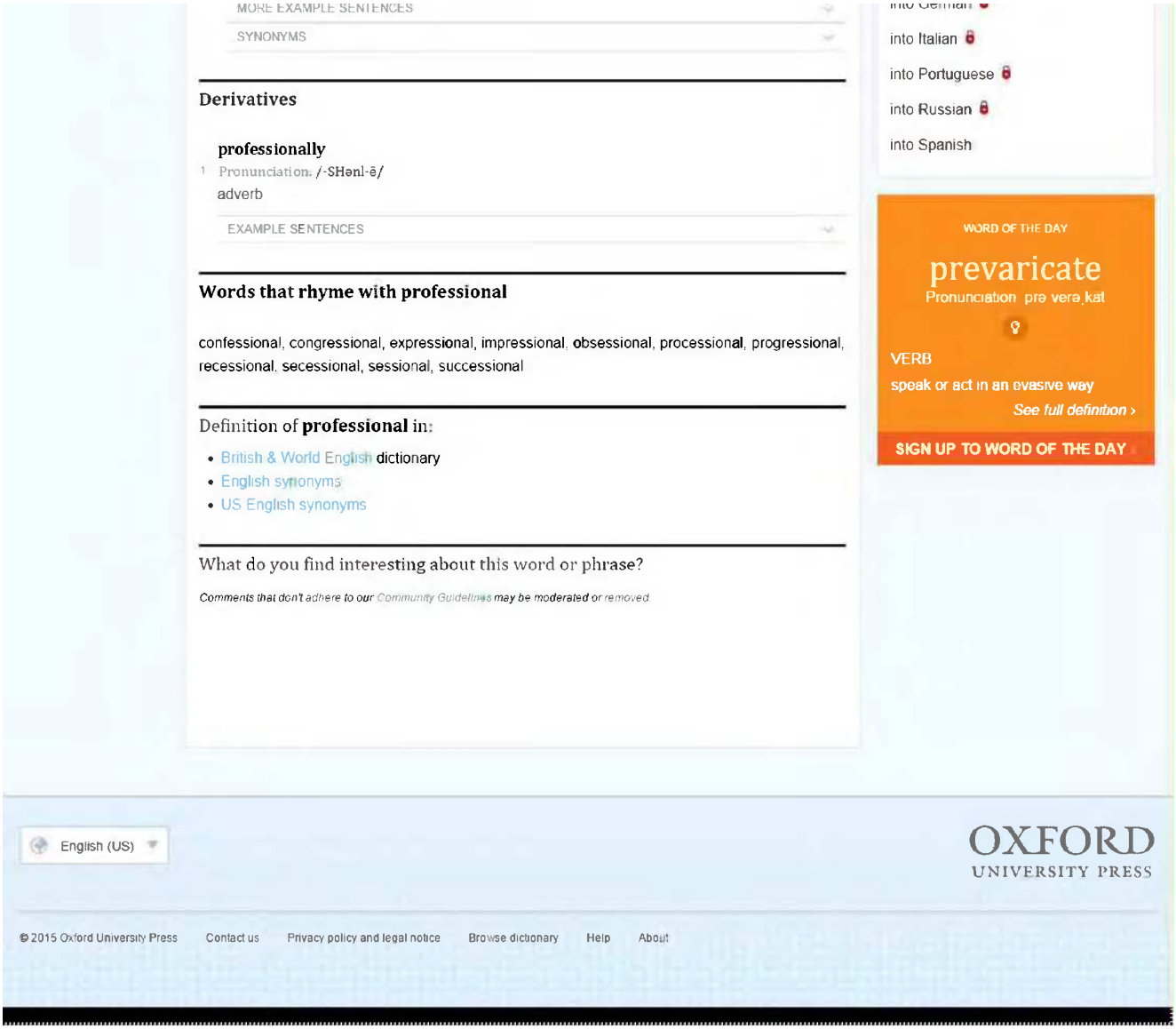
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SYNONYMS
Derivatives
professionally
1 Pronunciation: /-SHənl-ē/
adverb
EXAMPLE SENTENCES
Words that rhyme with professional
confessional, congressional, expressional, impressional, obsessional, processional, progressional, recessional, secessional, sessional, successional
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US English synonyms
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WORD OF THE DAY
prevaricate
Pronunciation: prə verə kāt
VERB
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There are 3 main definitions of *pro* in English: 1 2 3

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pro 1

Syllabification: pro
Pronunciation: /prō/

noun (plural **pros**)

informal

A professional, especially in sports:
'a tennis pro'

MORE EXAMPLE SENTENCES

adjective

Back to top

(Of a person or an event) professional:
'a pro golfer'

MORE EXAMPLE SENTENCES

Origin

Mid 19th century: abbreviation.

Words that rhyme with pro

aglow, ago, alow, although, apropos, art nouveau, Bamako, Bardot, beau, Beaujolais Nouveau, below, bestow, blow, bo, Boileau, bons mots, Bordeaux, Bow, bravo, bro, cachepot, cheerio, Coe, crow, Defoe, de trop, doe, doh, dos-à-dos, do-si-do, dough, dzo, Flo, floe, flow, foe, foreknow, foreshow, forgo, Foucault, froe, glow, go, good-oh, go-slow, grow, gung-ho, Heathrow, heave-ho, heigh-ho, hello, ho, hoe, ho-ho, jo, Joe, kayo, know, lo, low, maillot, malapropos, Marceau, mho, Miró, mo, Mohs, Monroe, mot, mow, Munro, no, Noh, no-show, oh, oho, outgo, outgrow, owe, Perrault, pho, po, Poe, quid pro quo, reshow, righto, roe, Rouault, row, Rowe, sew, shew, show, sloe, slow, snow, so, soh, sow, status quo, stow, Stowe, strow, tally-ho, though, throw, tic-tac-toe, to-and-fro, toe, touch-and-go, tow, trow, undergo, undersow, voe, whacko, whoa, wo, woe, Xuzhou, yo, yo-ho-ho, Zhengzhou, Zhou

Definition of **pro** in:

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- English synonyms
- Spanish dictionary

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MORE ON PRO

Nearby words

prizewinner
p.r.n.
pro
pro- 1
pro- 2

See also

pro-1 in US English
pro-2 in US English
pro-am in US English
pro-ana in US English
pro Deo in US English
pro tem in US English

Result list for **pro**

MORE WORDS IN THIS CATEGORY

big air
breakaway
calisthenics

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Syllabification: pro
Pronunciation: /prō/

noun (plural **pros**)

(usually **pros**)
An advantage of something or an argument in favor of a course of action:
'the pros and cons of joint ownership'

MORE EXAMPLE SENTENCES

preposition& adverb Back to top

In favor of:
(AS PREPOSITION) *'they were pro the virtues of individualism'*

MORE EXAMPLE SENTENCES

Origin

Late Middle English (as a noun): from Latin, literally 'for, on behalf of'.

Definition of **pro** in:

- British & World English dictionary
- English synonyms
- Spanish dictionary

There are 3 main definitions of *pro* in English: 1 2 3

PRO 3

Syllabification: PRO
Pronunciation:

abbreviation

1 Public relations officer.

1.1 *British* Public Record Office.

Definition of **pro** in:

- British & World English dictionary

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captaincy

catch-up

gene

Top 1000 frequently used words

Syllabification: gene

Pronunciation: /jēn/

Definition of *gene* in English:

noun

Biology

1 (In informal use) a unit of heredity that is transferred from a parent to offspring and is held to determine some characteristic of the offspring:
'proteins coded directly by genes'

MORE EXAMPLE SENTENCES

1.1 (In technical use) a distinct sequence of nucleotides forming part of a chromosome, the order of which determines the order of monomers in a polypeptide or nucleic acid molecule which a cell (or virus) may synthesize.

EXAMPLE SENTENCES

Origin

Early 20th century: from German *Gen*, from *Pangen*, a supposed ultimate unit of heredity (from Greek *pan-* 'all' + *genos* 'race, kind, offspring').

MORE

Words that rhyme with gene

Aberdeen, Amin, aquamarine, baleen, bean, been, beguine, Benin, between, canteen, careen, Claudine, clean, contravene, convene, cuisine, dean, Dene, e'en, eighteen, fascine, fedayeen, fifteen, figurine, foreseen, fourteen, Francine, gean, glean, gombeen, green, Greene, Halloween, intervene, Janine, Jean, Jeannine, Jolene, Kean, keen, Keene, Ladin, langoustine, latrine, lean, limousine, machine, Maclean, magazine, Malines, margarine, marine, Mascarene, Massine, Maxine, mean, Medellín, mesne, mien, Moline, moreen, mujahedin, Nadine, nankeen, Nazarene, Nene, nineteen, nougatine, obscene, palanquin, peen, poteen, preen, quean, Rabin, Racine, ramin, ravine, routine, Sabine, saltine, sardine, sarin, sateen, scene, screen, seen, serene, seventeen, shagreen, shebeen, sheen, sixteen, spleen, spring-clean, squireen, Steen, submarine, supervene, tambourine, tangerine, teen, terrine, thirteen, transmarine, treen, tureen, Tyrrhene, ultramarine, umpteen, velveteen, wean, ween, Wheen, yean

Definition of **gene** in:

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Nearby words

gender reassignment
gender role
genealogical
genealogical tree
gene

See also

gene map in US English
gene doping in US English
gene pool in US English
Kelly, Gene in US English
Autry, Gene in US English
Krupa, Gene in US English

Result list for **gene**

MORE WORDS IN THIS CATEGORY

antisense
backcross
chimera
cis 1

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gene map

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into Chinese

into French

into German

into Italian

into Portuguese

into Russian

into Spanish

WORD OF THE DAY

prevaricate

Pronunciation prə'verə,kāt

VERB

speak or act in an evasive way

See full definition ›

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